Effect of Severe Undernutrition in Early Life on Growth, Brain Size and Composition in Adult Rats[1]

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ABSTRACT A study was undertaken to determine the effect of varying periods of undernutrition in postnatal life on the size and chemical composition of the brain in rehabilitated adult animals. Four groups of male albino rats were drawn from litters of 16 pups nursed by an undernourished dam. One group was weaned at 3 weeks to a diet of 18% casein. Three similar groups were fed a 3% protein diet until 5, 7 and 9 weeks of age. A diet of 18% protein was then fed until the rats were 19 weeks old. The growth, and brain size and composition of these nutritionally deprived groups were compared with those of littermates adequately nourished from birth. Undernutrition during suckling caused a degree of stunting of body size that was only partially reversed by nutritional rehabilitation. Brain size and brain DNA were depressed by undernutrition in the preweaning period but were not affected further by deprivation in the postweaning period. They did not respond to an adequate diet. Brain size was less affected than total body size. Cholesterol levels of brain were significantly and irreversibly depressed if undernutrition occurred through 5 weeks of age but responded to earlier rehabilitation. Phospholipid phosphorus and RNA content of brain were similar in all groups. The chemical composition of the smaller brains of the deprived animals was not significantly different on the basis of percentage composition than that of the larger brains of the well-nourished animals.

Studies of children who have suffered severe protein-calorie malnutrition in early infancy suggest that brain size and intellectual development suffer a degree of damage that is refractory to subsequent rehabilitation (1–4). Results of these studies are difficult to interpret because of the interaction of antecedent and subsequent environmental, cultural, and nutritional determinants. Thus it has become necessary to rely on animal studies where more precise controls are possible to determine whether a relationship exists between the plane of nutrition in early life and subsequent brain development.

Previous research has assessed separately the effect of undernutrition in the preweaning and postweaning periods. Although there have been reports of the effect of underfeeding from birth into the postweaning period on behavior in rehabilitated adult animals (5), little attention has been given to its effects on brain growth and composition.

Investigators who have fed pups in large litters or who have restricted feeding times have found that the growth of the brain is significantly depressed by undernutrition during suckling (6–8). In addition, Dobbing (9) noted a depressed cholesterol content, and Culley and Mertz (8), lower cholesterol, cerebroside and phospholipid values in deprived animals than in adequately fed controls. The difference in the case of the phospholipid values was not, however, significant. Winick and Noble (10) observed that the lower brain size and reduced brain DNA content resulting from undernutrition during suckling had not responded to 16 weeks of rehabilitation. Their findings are not corroborated by Benton et al. (7) who showed that rehabilitation initiated at weaning had eliminated differences in brain size, brain cholesterol, and phospholipid phosphorus content by 6 weeks of age.

Dobbing and Widdowson (11) reported that undernutrition initiated at weaning had no effect on brain size. They observed lower phospholipid phosphorus and cholesterol values which were restored to normal by adequate nutrition beginning at 11 weeks. Similarly, rats subjected to undernutrition from 5 to 8 weeks and from 9 to

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12 weeks had a brain size and DNA content at 19 weeks comparable to those of adequately fed controls (7).

Undernutrition from birth through 9 weeks before rehabilitation from 9 to 15 weeks, resulted in a lower brain DNA, cholesterol, phospholipid phosphorus and cerebroside content.[3]

In general, previous studies have shown that undernutrition during suckling has a permanent depressing effect on brain size and on some aspects of brain composition, whereas undernutrition occurring in the postweaning period has a smaller effect which can be eliminated by nutritional rehabilitation.

The purpose of the present study was twofold: first, to determine the effect of severe nutritional deprivation from birth through varying periods after weaning before rehabilitation, on growth, brain size and composition in rats; and second, to determine whether there is a critical period in the development of the central nervous system of the rat after which nutritional rehabilitation is ineffective in reversing any detrimental effects of earlier undernutrition.

EXPERIMENTAL PROCEDURE

Twenty-four pups born within 12 hours of one another to Manor Farm Hyrac strain albino dams fed a laboratory ration of 23% protein content during gestation were pooled and distributed at random to a control group (C) of 8 pups and a deprived group (D) of 16 pups. The control group was nursed by a mother fed a diet of approximately 18% protein; those in the deprived group by a dam changed to a diet of approximately 8% protein at 2 days postpartum. At 21 days all pups were weaned and the females discarded. Two to four males from group C were fed the 18% protein diet for 16 weeks. The males from group D were distributed at random to 4 experimental groups and fed as outlined in table 1. This procedure was repeated 6 times to provide 12 animals per experimental group.

The composition of the experimental diets is shown in table 2. Diets were fed ad libitum. The rats were housed in individual cages in a room with controlled temperature, artificial ventilation and artificial light regulated to provide 14 hours of light each day. To assure consistent treatment, only one person handled the animals for routine care and weekly weighings.

At 19 weeks of age, following 3 weeks of a series of behavioral tests, the animals were killed by mild ether anaesthesia and removal of blood by heart puncture. The head was chilled and the brain was blotted free of excess blood before being removed by severing it at the foramen magnum and olfactory lobes. The weighed, chilled brain was homogenized in a Virtis-45 tissue homogenizer for 2 minutes. Two 5-ml ali-

[3] Culley, W. J., E. T. Mertz, R. O. Lineburger and R. E. Gotts 1967 Effect of early undernutrition on brain composition of adult rats. Federation Proc., 26: 519 (abstract).

TABLE 1
Experimental design

Group	Age of rats [1] — Diet during postweaning period: 3% protein	Age of rats [1] — Diet during postweaning period: 18% protein	No. of rats [2]
	weeks	*weeks*	
		Control (C) (8 pups/litter; mother fed 18% protein diet during suckling)	
C	—	3–19	12
		Deprived (D) (16 pups/litter; mother fed 8% protein diet during suckling)	
D-3	—	3–19	12
D-5	3–5	5–19	12
D-7	3–7	7–19	11
D-9	3–9	9–19	9

[1] Age from birth.
[2] One rat in group D-7 and 3 rats in group D-9 died before rehabilitation.

TABLE 2

Composition of experimental diets

	Control	Low protein for lactating mother	Low protein for deprived weanling rats
	g	*g*	*g*
Casein	18	8	3
Cornstarch	68	78	83
Vegetable oil	10	10	10
Salt mixture USP XIV [1]	4	4	6
Vitamin fortification mix [2]	2	2	4

[1] Composition of salt mixture: (in milligrams) cupric sulfate, 0.48; calcium carbonate, 68.6; calcium citrate, 308.3; calcium biphosphate, 112.8; magnesium carbonate, 35.2; magnesium sulfate, 38.3; potassium chloride, 124.7; dibasic potassium phosphate, 218.8; sodium cholide, 77.1; mixture of ferric ammonium sulfate (94.33), manganese sulfate (1.24), ammonium aluminate (0.57), potassium iodide (0.25), sodium fluoride (3.13), 16.2.

[2] Composition: (in g/kg) vitamin A, 4.5; vitamin D, 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; calcium pantothenate, 3.0; biotin, 0.020; folic acid, 0.050; and vitamin B_{12}, 0.00135.

quots of the brain homogenate were used for duplicate RNA analyses according to the method of Munro and Fleck (12) and for DNA analyses on a hot acid extract by the method of Ogur and Rosen (13). Both methods are based on ultra violet absorption at 260 mμ.

Lipids were extracted from a 10-ml aliquot of brain using 2 extractions with 95% ethanol and 2 with methanol:chloroform (3:1, v/v).

Duplicate 2-ml aliquots of the 50-ml lipid extract were evaporated to dryness, and redissolved in 6-ml 0.1 N potassium acetate in absolute alcohol. These samples were saponified with 0.2 ml 33% KOH and assayed for cholesterol according to the method of Koval (14). Duplicate 2-ml samples of the lipid extract were digested with 0.5 N sulphuric acid for 3 hours and oxidized with 5 drops of 33% hydrogen peroxide for 1.5 hours in a sand bath at 150 to 160°. Phospholipid phosphorus was determined on these samples by a modification of the method of Fiske and Subbarow (15).

A one-way analysis of variance was carried out on the data for growth, brain size and chemical composition. For those variables showing a significant *F*-ratio Duncan's multiple range test (16) was applied to determine which values differed from control values or from each other.

RESULTS AND DISCUSSION

Growth curves for each of the experimental groups compared with adequately nourished controls are shown in figure 1. At weaning, rats in the deprived groups weighed only 28% as much as those in the control group—considerably less than the 48 and 51% reported by others who had

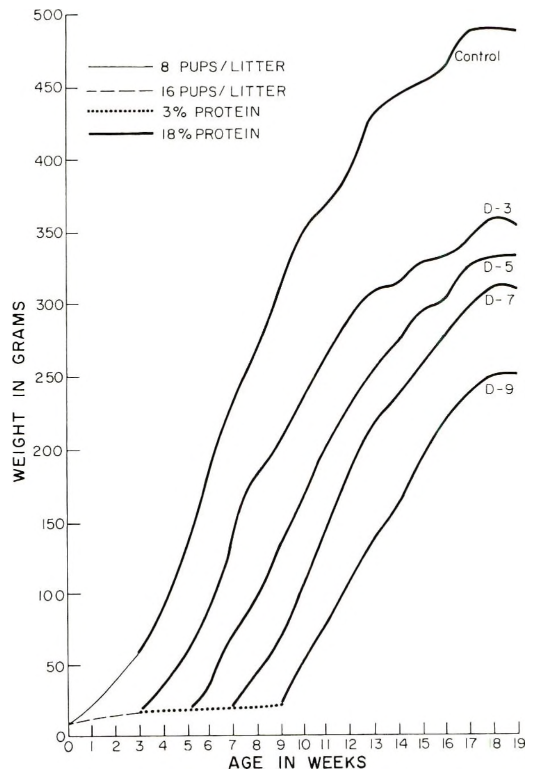
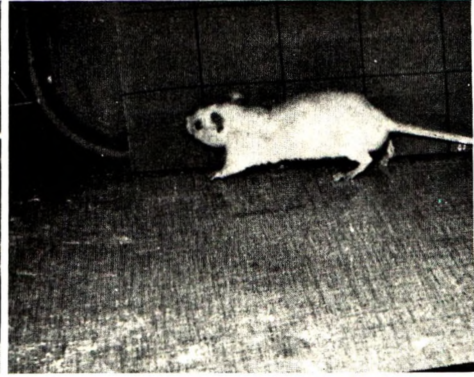


Fig. 1 Growth curves of rats subjected to nutritional deprivation for 3, 5, 7 or 9 weeks following birth before rehabilitation, compared with rats adequately nourished from birth.

GROUP C

GROUP D-3

GROUP D-5



GROUP D-7

GROUP D-9

Fig. 2 Representative rats from each experimental group at 9 weeks of age following zero, 3, 5, 7 and 9 weeks of severe undernutrition. Average weights for each group were 311, 205, 134, 69 and 19 g, respectively (scale of background 1 square = 5 cm × 5 cm).

fed large litters with a well-nourished dam (10, 17). The weaning weight (17–20 g) of the deprived animals was maintained throughout the period of deprivation following weaning when a diet of 3% protein was fed. The relative sizes of representative animals at 9 weeks of age are illustrated in figure 2. At this age, weights of groups D-3, D-5, D-7 and D-9 which had experienced 3, 5, 7 or 9 weeks of undernutrition represented 65, 46, 22 and 6% of the weight of control group, respectively. For all groups the growth curve began to level off at approximately the same age (16 to 18 weeks) regardless of weight, suggesting that although animals deprived early in life might continue to grow slowly they would never compensate completely for the deficit in body weight resulting from undernutrition at an early age. The weights of animals in groups D-3, D-5, and D-7 represented 72, 69, 63% of that of controls at 19 weeks of age, differences significant at the 1% level of confidence. Group D-9 weighed 52% of that of control group and its growth curve established a plateau at a weight significantly less than all other groups.

These data show that rats can recover to a limited extent from nutritional deprivation sufficiently severe to restrict growth during suckling to less than one-third of normal weights and to prevent further growth up to 6 weeks after weaning. The degree of stunting increases the longer the period of postweaning deprivation. Differences between control rats and all experimental animals remained significant ($P < 0.01$) from 2 to 19 weeks.

Food consumption of rats fed the 3% protein diet ranged from 2 to 4 g per day, and that of rats fed the control diet of 18% protein increased from 7 to 20 g with increase in size of the animal. All rats survived the suckling period but 4 rats, three of which were from the same pooled litter died after 7 weeks of deprivation. Diarrhea always preceded death.

Five out of nine rats in group D-9 developed an opacity of the cornea resembling that described in vitamin A deficiency. No other classical vitamin deficiency symptoms were observed. The appearance of vitamin A deficiency symptoms may represent a failure to mobilize hepatic reserves or a failure of absorption in a protein deficiency rather than a vitamin A deficiency per se (18). The diet provided 1800 IU vitamin A/100 g. Even for the 20-g rats consuming only 2 g of diet a day, the intake of 36 IU is in excess of the 10 IU/day recommended for an animal of this size.

Brain weights and the relationship between brain weight and body size is shown in table 3. Undernutrition during suckling caused a significant ($P < 0.01$) depression in the size of the brain, which was not reversed by adequate nutrition introduced at weaning. The brains of rats undernourished for 3, 5, or 7 weeks after birth reached 87% of the size of controls but did not differ from one another. Brains of rats in group D-9 were significantly smaller ($P < 0.05$) than those of rats deprived for only 3 or 5 weeks. Thus, rehabilitation initiated as late as 7 weeks after deprivation from birth may stimulate a degree of recovery in brain size that is not possible after 9 weeks of deprivation. The depression in brain size resulting from deprivation during suckling is at least partially irreversible.

The brains of rats in deprived groups were smaller, but their body growth was still more stunted so that the brains of the

TABLE 3

Means, standard deviations and analysis of variance for brain weight and its relation to body weight at 19 weeks of age

	Group					
	Control	D-3	D-5	D-7	D-9	F ratio
Brain wt, g	1.95 ± 0.09 [1]	1.70 ± 0.10	1.72 ± 0.09	1.68 ± 0.06	1.60 ± 0.12	21.14 [2]
Brain wt, % of body wt	0.41 ± 0.03	0.48 ± 0.03	0.51 ± 0.03	0.55 ± 0.06	0.65 ± 0.10	31.26 [3]

[1] Mean ± SD.
[2] Control > D-3, D-5, D-7, or D-9 $P < 0.01$
D-3, D-5 > D-9 $P < 0.05$
[3] Control < D-3, D-5, D-7 or D-9 $P < 0.01$
D-3, D-5, D-7 < D-9 $P < 0.01$

rats deprived the longest represented an increasing proportion of total body weight. This finding confirms observations of earlier workers (11, 19) that the brain is protected to a greater extent than the total body from the effects of undernutrition. Rats deprived for 9 weeks had brains representing a significantly higher percentage of body weight than those deprived for 3, 5, or 7 weeks. These, in turn, were significantly higher than for control rats.

Brain composition data in table 4 on the DNA, RNA, cholesterol and phospholipid phosphorus content and concentration show that undernutrition following birth has differential effects on each of these components.

DNA values, considered indicative of cell number (20) are significantly lower in brains of all groups of rats deprived during suckling than in brains of adequately fed controls. That DNA content is not lower following longer periods of deprivation, and remains the same regardless of timing of rehabilitation, indicates the critical period for cell division is in the first 3 weeks of life, deprivation beyond this point has no further detrimental effect, and adequate nutrition at weaning does not stimulate brain cell division. This is in keeping with the findings of Winick and Noble (21) that cell proliferation in normal rat brain is complete by 3 weeks and that lower DNA values result when deprivation occurs during suckling, but not when it occurs later (10).

DNA per gram of brain tissue did not differ among groups, showing that differences in brain size were a function of differences in cell number rather than cell size. This is substantiated by a high correlation coefficient ($r = 0.71$) between brain weight and DNA levels.

Neither RNA content nor concentration in brain differed significantly among groups although the control groups had highest values and group D-9, the lowest. RNA-to-DNA ratios of approximately one, are in accord with those previously reported for brain tissue (22).

Groups C and D-3, whose brain cholesterol level was similar, had values significantly higher than those in brains of groups deprived for longer periods. It thus appears that rehabilitation initiated before 5 weeks of age can promote cholesterol deposition, whereas rehabilitation after 5 weeks cannot compensate sufficiently for the effect of undernutrition in the suckling period. Group D-3 had a cholesterol content 93% of that of group C, whereas that of other deprived groups was less than 75%. Although the concentration of cholesterol in the smaller brains of deprived animals is lower, it is not significantly different than in well-nourished animals. Thus it appears that undernutrition has no specific effect on cholesterol deposition. The finding that cholesterol deposition is less in animals de-

[4] Diets were obtained from Nutritional Biochemicals Corporation, Cleveland.
[5] See footnote 3.

TABLE 4

Mean values, standard deviations and analysis of variance for DNA, RNA, phospholipid phosphorus and cholesterol content and concentration of brains

Groups	C	D-3	D-5	D-7	D-9	
No. of rats	11	12	12	11	9	*F* ratio
			mg/brain			
DNA [1]	3.34 ± 0.15 [2]	2.69 ± 0.29	2.83 ± 0.34	2.80 ± 0.27	2.79 ± 0.25	8.80 [3a]
RNA	3.06 ± 0.51	2.75 ± 0.38	2.78 ± 0.22	2.79 ± 0.23	2.57 ± 0.27	2.38
Cholesterol	36.9 ± 3.8	34.1 ± 3.5	29.6 ± 4.9	29.8 ± 6.4	29.0 ± 3.4	6.23 [3b]
Phospholipid phosphorus	3.49 ± 0.55	3.31 ± 0.74	3.59 ± 0.45	3.79 ± 0.79	3.17 ± 0.58	1.46
			mg/g brain			
DNA	1.72 ± 0.09	1.56 ± 0.15	1.68 ± 0.14	1.61 ± 0.15	1.77 ± 0.17	3.18
RNA	1.56 ± 0.23	1.61 ± 0.18	1.63 ± 0.09	1.64 ± 0.15	1.62 ± 0.11	0.38
Cholesterol	18.8 ± 1.7	20.0 ± 1.7	17.3 ± 2.9	17.4 ± 4.0	18.2 ± 2.6	1.96
Phospholipid phosphorus	1.78 ± 0.28	1.94 ± 0.42	2.09 ± 0.24	2.21 ± 0.44	1.96 ± 0.25	2.48

[1] n = 11, 11, 11, 10, 8 because of technical error in one day's sample.
[2] Mean ± SD.
[3] Significance of differences among group means based on Duncan's multiple range test (16).
a. Total DNA. C > D-3, D-5, D-7 or D-9 — $P < 0.01$
b. Total cholesterol. C > D-5, D-7, D-9 — $P < 0.01$
D-3 > D-5, D-7, D-9 — $P < 0.05$

prived up to 5 weeks corresponds to findings, using radioactive precursors of cholesterol (23), that cholesterol synthesis is most rapid in the first 3 weeks of life, and virtually complete by 5 weeks.

Neither the total phospholipid phophorus content nor its concentration on a percentage basis in the brains of the deprived animals differed significantly from those of the controls; nor was any trend evident related to the severity of the deprivation. Our results with animals rehabilitated for 9 weeks do not confirm those of Culley and Mertz [8] who found depressed values after 9 weeks of deprivation and 6 weeks of rehabilitation. Our failure to show similar effects on total phospholipid phosphorus and cholesterol, both components of the myelin sheath, may be the result of the phospholipid portion being more responsive to nutritional rehabilitation than the cholesterol-containing portion (24).

The data on the chemical composition of the brain show that although cell number is reduced with undernutrition during suckling and myelination, as measured by the cholesterol values, and is inhibited by 5 weeks of deprivation, the chemical makeup of the brain on a per unit basis is similar in control and deprived animals. Thus the critical period for cell division in the brain is in the first 3 weeks of life and for cholesterol deposition, the first 5 weeks of life. Undernutrition at this time inhibits both these processes and subsequent nutritional rehabilitation is ineffective in reversing the damage.

Our findings on brain size and composition are comparable to those of other investigators who studied a milder degree of undernutrition which allowed almost twice the weight gain during suckling (8, 17).

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Antithiamine Activity of Dimethialium and Its Mode of Action [1]

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ABSTRACT The antithiamine activity of dimethialium, a thiamine analogue having a methyl group in place of the hydroxyethyl group in the thiazole moiety, has been demonstrated in nutritional assays using rats or chicks. The antagonism was evident only when thiamine was administered orally. The inhibition was competitive with thiamine or thiamine propyl disulfide. The latter counteracted the antithiamine activity of dimethialium two to four times more effectively than did thiamine. Thiamine uptake in vitro by Ehrlich ascites carcinoma cells was inhibited competitively by dimethialium, whereas the uptake of thiamine tetrahydrofurfuryl disulfide was not affected. Dimethialium inhibited thiaminokinase competitively with thiamine but showed little or no effect upon pyruvate decarboxylation by carboxylase or pyruvate oxidase complexes. All these results demonstrate that dimethialium is a typical but relatively weak antithiamine whose antagonistic activity is far less than that of pyrithiamine and comparable to that of amprolium. The present data also support the view that the primary cause of the antithiamine action is its interference with intestinal absorption or cellular uptake of thiamine.

Among the many thiamine antagonists hitherto discovered, pyrithiamine and oxythiamine have been successfully used for elucidation of the mode of thiamine action (1, 2). Cuckler et al. (3) and others (4) found that a thiamine analogue, amprolium,[3] showed an effective anticoccidial activity which was nullified by thiamine. Later Ott et al. (5) reported that amprolium exerted an antithiamine activity in chick growth assay. During the screening studies on thiamine-related compounds we found that dimethialium,[4] the thiamine analogue having a methyl group at the 5-position of the thiazole moiety, showed a typical antithiamine action in rat growth experiments. Independently of our investigations, Hiraoka et al. (6) also observed antithiamine activity of this compound in the growth of *Lactobacillus fermenti.* The present report describes the thiamine antagonism of dimethialium in several biological systems including rat and chick growth assays, thiamine uptake by Ehrlich ascites carcinoma cells and in vitro enzymatic investigations. The possible mode of action is also discussed.

METHODS

Rat growth assay. Weanling male rats of the Sprague-Dawley strain were kept individually in metal cages with screen-bottoms and fed a thiamine-deficient diet ad libitum. The animal room was maintained at 24° with controlled humidity. The diet consisted of the following: (in percent) vitamin-free casein, 18.0; sucrose, 67.6; cottonseed oil,[5] 8.0; salts (7), 4.0; powdered cellulose, 1.5; DL-methionine, 0.3; choline chloride, 0.1; and vitamin mixture,[6] 0.5. For prophylactic assays, the rats received thiamine, dimethialium, or both, dissolved in 0.2 ml of water, orally by stomach tube each day or every other day throughout the assay extending 3 weeks. The dosing regimen is shown in

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[2] Kyoto Herbal Garden.

[3] Coccidiostat obtainable from Merck and Company, Inc., Rahway, New Jersey.

[4] Dimethialium has the structure of 3-(2-methyl-4-aminopyrimidin-5-yl)methyl-4,5-dimethyl thiazolium. The compound is used as coccidiostat; Actonate, Takeda Chemical Ind., Ltd., Osaka, Japan.

[5] The oil contained vitamin A palmitate (2000 IU), vitamin D_2 (200 IU) and *dl-α*-tocopheryl acetate (10 mg) per 8 ml.

[6] Contained in mg per 0.5 g of mixture: riboflavin, 0.8; pyridoxine·HCl, 0.5; niacin, 5; Ca pantothenate, 4; biotin, 0.04; folic acid, 0.2; vitamin B_{12}, 0.003; *p*-aminobenzoic acid, 10; inositol, 10; ascorbic acid, 20; menadione, 0.5; glucose was added to the mixture to make 0.5 g.

table 1 and the body weight gain was estimated from the 3-week assay period. In curative assays the rats were divided into groups of 7 to 8 rats after a depletion period of about 3 weeks and then were given thiamine, dimethialium, or both, by feeding or intraperitoneal injection. Figure 1 illustrates a typical curative assay. The details of the dosage regimen are shown in table 2 and figures 1 and 2. The growth response is expressed as weekly gain in body weight obtained during the

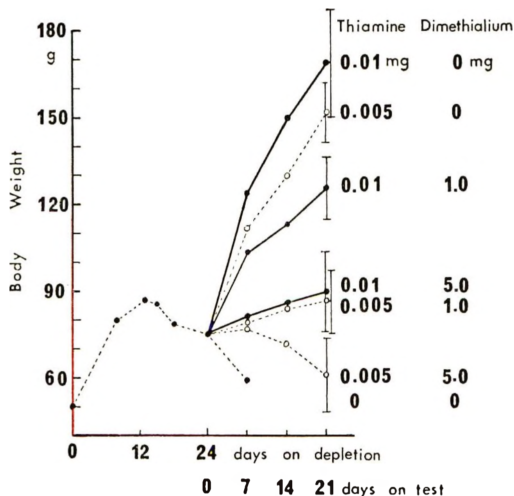


Fig. 1 Antithiamine activity of dimethialium in rat curative assay. The numbers show the daily doses of the vitamin and antagonist; both were given orally by stomach tube.

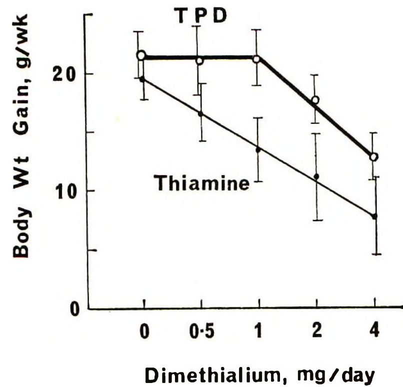


Fig. 2 Antithiamine activity of dimethialium against thiamine propyl disulfide (TPD) in rat curative assay. Thiamine (5 μg) and TPD (5 μg as thiamine·HCl) with the indicated amounts of dimethialium were given orally by stomach tube. Data are expressed as mean ± SD (n = 7).

3-week assay period. Rats in some groups were given thiamine propyl disulfide[7] in place of thiamine (fig. 2).

Chick growth assay. One-day-old White Leghorn chicks were kept in an electrically heated battery and fed a chick starter diet

[7] Thiamine propyl disulfide (TPD) is 2-(2-methyl-4-aminopyrimidin-5-yl)methylformamido-5-hydroxy-2-penten-3-yl propyl disulfide (refer to Merck Index, ed. 7). Thiamine tetrahydrofurfuryl disulfide (TTFD) is the tetrahydrofurfuryl homologue of thiamine propyl disulfide. The following reviews discuss the chemical and biological properties of these compounds: Nutr. Rev., *16:* 336, 1958; Nutr. Rev., *18:* 181, 1960; Ann. Rev. Biochem., *24:* 419, 1955; and Vitamins Hormones, *21:* 69, 1963. The thiamine alkyl disulfides were obtained from Takeda Chemical Industries, Ltd., Osaka, Japan.

TABLE 1

Antithiamine activity of dimethialium in rat prophylactic assay

Group no.	Dose		No. of rats dead/total	Body wt gain [2]
	Thiamine	Dimethialium		
	mg/rat/day	*mg* [1]*/rat/day*		*g/rat/wk*
1	—	—	0/8	3.0 ± 1.7 [3]
2	0.01	—	0/8	28.0 ± 1.7
3	0.01	0.01	0/8	26.0 ± 4.7
4	0.01	0.1	0/8	24.0 ± 5.3
5	0.01	1	0/7	22.7 ± 4.0
6	0.01	10	0/8	8.3 ± 3.0
7	0.01	100	8/8	—
8	0.1	—	0/7	31.3 ± 5.3
9	0.1	0.1	0/8	32.3 ± 4.7
10	0.1	1	0/7	31.7 ± 3.0
11	0.1	10	0/7	29.0 ± 5.3

[1] Milligram equivalents calculated as thiamine·HCl.
[2] Significant ($P < 0.05$) in t test, group 2 versus 5. Not significant ($P > 0.05$), group 2 versus 3, 2 versus 4, 8 versus 9, 8 versus 10, and 8 versus 11.
[3] Mean ± SD.

TABLE 2

Effect of administration route on the antithiamine activity of dimethialium in rat curative assay

Group no.	Dose and administration route		No. of rats dead/total	Mean survival time	Body wt gain [1]
	Thiamine	Dimethialium			
	mg/rat/day	*mg [2]/rat/day*		*days [3]*	*g/rat/wk*
1	—	—	5/5	15.4	—
2	—	2.5 per os [4]	7/7	17.0	—
3	—	2.5 ip [5]	7/7	12.7	—
4	0.005 per os	—	0/7	> 21	11.0 ± 2.4 [6]
5	0.005 per os	2.5 per os	0/7	> 21	4.5 ± 2.4
6	0.005 per os	2.5 ip	0/7	> 21	7.1 ± 1.9
7	0.005 ip	—	0/7	> 21	12.6 ± 3.8
8	0.005 ip	2.5 per os	0/7	> 21	11.1 ± 5.6
9	0.005 ip	2.5 ip	0/7	> 21	12.1 ± 3.5

[1] Significant in t test, $P < 0.005$ for group 4 versus 5, $P < 0.01$ for group 4 versus 6, $P < 0.05$ for group 5 versus 6. Not significant ($P > 0.5$) for group 7 versus 8, 7 versus 9, and 8 versus 9.
[2] Milligram equivalents calculated as thiamine·HCl.
[3] A 21-day test period.
[4] Per os by stomach tube.
[5] Intraperitoneal injection.
[6] Mean ± SD.

TABLE 3

Antithiamine activity of dimethialium in chick growth

Group no.	Supplement to deficient diet		No. of chicks dead/poly.[1]/total	Body wt gain
	Vitamin	Dimethialium		
	mg/kg diet	*mg [2]/kg diet*		*g/chick/wk*
		Thiamine hydrochloride		
1	—	—	4/5/10	−20 ± 3.5 [3]
2	0.2	—	2/5/10	−19 ± 4.5
3	0.4	—	0/4/10	−15 ± 2.1
4	0.8	—	0/0/7	26 ± 6.7
5	4.0	—	0/0/10	63 ± 5.4
6	0.8	40	0/2/10	4 ± 10.0
7	0.8	80	0/2/9	− 2 ± 8.1
8	0.8	160	0/4/10	− 8 ± 6.8
9	0.8	320	0/4/10	− 9 ± 5.7
		Thiamine propyl disulfide [2]		
10	0.2	—	2/4/10	−17 ± 6.6
11	0.4	—	0/2/10	− 8 ± 4.5
12	0.8	—	0/0/9	28 ± 10.8
13	4.0	—	0/0/7	64 ± 6.6
14	0.8	40	0/0/10	20 ± 9.6
15	0.8	80	0/2/10	9 ± 14.8
16	0.8	160	0/4/10	− 2 ± 9.2
17	0.8	320	0/3/7	− 9 ± 6.2

[1] Polyneuritis.
[2] Milligram equivalents calculated as thiamine·HCl.
[3] Mean ± SD.

for the first 3 days. A thiamine-deficient diet, consisting of: (in percent) vitamin-free casein, 20; gelatin, 10; sucrose, 48.6; powdered cellulose, 5.0; corn oil,[8] 4.5; calcium gluconate, 2.5; glycine, 2.0; L-arginine·HCl, 0.5; DL-methionine, 0.2; choline chloride, 0.2; salts (7), 6.0; and vitamin mixture,[9] 0.5, was fed for the next 3 days. The chicks were then weighed, divided into groups of 7 to 10 chicks and fed the supplemented diets for 7 days (tables 3 and 4). In addition to body weight gain, the incidence of polyneuritis and mortality

[8] The oil contained/4.5 ml: vitamin A palmitate, 2000 IU; vitamin D_2, 200 IU; and *dl-α*-tocopheryl acetate, 10 mg.
[9] See footnote 6.

TABLE 4
Effect of administration route of thiamine on the antithiamine activity of dimethialium in chick growth

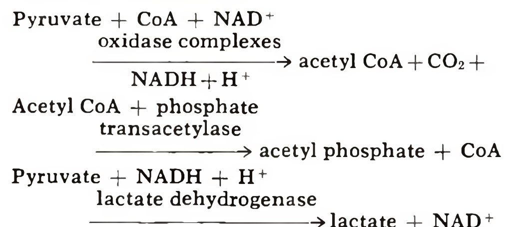
Group no.	Dose		Diet intake	No. of chicks dead/poly.[1]/total	Body wt gain [2]
	Thiamine	Dimethialium			
	mg/kg diet	*mg [3]/kg diet*	*g/chick/day*		*g/chick/wk*
1	—	—	—	4/10/10	−23 ± 3.2 [4]
2	0.5	—	2.9	0/5/10	− 9 ± 5.5
3	1	—	7.5	0/0/10	45 ± 14.9
4	2	—	9.1	0/0/10	63 ± 7.6
5	1	25	8.2	0/0/10	53 ± 16.1
6	1	50	6.1	0/0/10	30 ± 14.3
7	1	100	4.9	0/2/10	17 ± 22.5
8	1	200	4.1	0/3/9	7 ± 16.9
	µg/chick/day (injection) [5]				
9	5	—	4.6	0/0/10	15 ± 6.0
10	10	—	6.7	0/0/10	39 ± 5.7
11	20	—	8.0	0/0/10	54 ± 6.3
12	10	25	6.4	0/0/8	31 ± 7.7
13	10	50	5.8	0/0/10	28 ± 4.6
14	10	100	7.1	0/0/10	32 ± 7.1
15	10	200	5.9	0/0/10	21 ± 4.7

[1] Polyneuritis.
[2] Significant ($P < 0.05$) for 3 versus 6, 10 versus 14, ($P < 0.025$) for 10 versus 12, ($P < 0.005$) for 3 versus 7, 10 versus 13, 10 versus 15, and 12 versus 15.
[3] Milligram equivalents calculated as thiamine·HCl.
[4] Mean ± SD.
[5] Subcutaneous injection.

was also recorded. The other experimental conditions were the same as those described by Ott et al. (5).

Thiamine uptake by Ehrlich ascites carcinoma cells. The effects of dimethialium on thiamine uptake by Ehrlich ascites carcinoma cells were studied by the method of Menon and Quastel (8). The cells corresponding to 150 to 200 mg as dry weight were added to Krebs-Ringer bicarbonate buffer, pH 7.4, containing glucose (10 mM). The vessels were gassed with 95% oxygen and 5% carbon dioxide mixture. After preincubation for 10 minutes at 37°, ^{14}C-thiamine was added with or without dimethialium to make a final volume of 3.0 ml of the reaction mixture. The suspension was incubated at 37° for 1 hour with gentle shaking. Then the cells were collected by centrifugation, washed twice with cold Krebs-Ringer phosphate buffer, pH 7.4 and finally resuspended in the same buffer. Aliquots (usually 0.1 ml) of the cell suspension and supernatant medium were directly pipetted into counting bottles containing 10 ml of PPO-POPOP-dioxane scintillation solution.[10] The radioactivity was counted by a liquid scintillation spectrometer.[11] The concentration of the radioactivity in the cell water was calculated on the basis of our preliminary data showing that 82.5% of the wet weight of the cells corresponded to the weights of cell water. Thus, the results (table 5) were expressed as the concentration ratio of the intracellular and extracellular radioactivities.

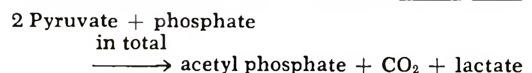
Enzymatic assays. Pyruvate oxidase complexes (pyruvate lipoate oxidoreductase, EC.1.2.4.1) were prepared from pig heart muscle (9), and *Escherichia coli* (10). The activity was assayed by the pyruvate dismutation method (11) by coupling with phosphotransacetylase (EC. 2.3.1.8) and lactate dehydrogenase (EC.1. 1.1.27), as follows:



$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{oxidase complexes}} \text{acetyl CoA} + CO_2 + \text{NADH} + H^+$$

$$\text{Acetyl CoA} + \text{phosphate} \xrightarrow{\text{transacetylase}} \text{acetyl phosphate} + \text{CoA}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$



$$2\ \text{Pyruvate} + \text{phosphate} \xrightarrow{\text{in total}} \text{acetyl phosphate} + CO_2 + \text{lactate}$$

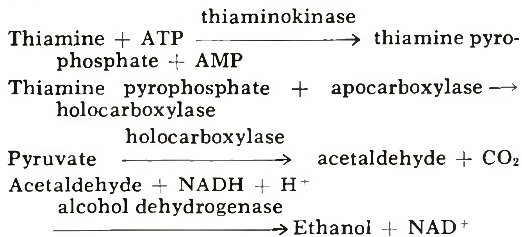
TABLE 5

Inhibitory effect of dimethialium on thiamine uptake by Ehrlich ascites carcinoma cells and its reversal by thiamine

Initial concn (medium)		Molar ratio of dimethialium/thiamine	Concn of ^{14}C-thiamine [1]			Inhibition
^{14}C-Thiamine	Dimethialium		Cell water	Medium	Concn ratio	
μM	μM		μM	μM		%
0.4	—	—	0.616	0.341	1.81	—
0.4	2	5	0.486	0.391	1.24	31
0.4	5	12.5	0.412	0.380	1.08	40
0.4	10	25	0.278	0.385	0.72	60
0.4	20	50	0.159	0.391	0.41	77
2	—	—	1.60	1.99	0.80	—
2	20	10	0.68	2.03	0.34	58
4	—	—	1.83	4.23	0.43	—
4	20	5	1.30	3.92	0.33	23
10	—	—	2.08	9.95	0.21	—
10	20	2	2.24	10.32	0.21	0

[1] After incubated for 1 hour at 37°.

Thiaminokinase (ATP:thiamine pyrophosphotransferase, EC.2.7.6.2) was purified from pig heart muscle and assayed by the method of Hamada and Koike.[12] The reaction mixture for thiaminokinase contained 50 μmoles of Tris-HCl buffer (pH 7.4), 5 μmoles of magnesium chloride, 3 μmoles of ATP, 0.02 or 0.1 μmole of thiamine and 0.63 mg of the thiaminokinase preparation. After incubation for 60 minutes at 37°, it was boiled for 2 minutes. The resultant supernatant was analyzed for the formation of thiamine pyrophosphate by Kaziro's method (12). The principle is based on spectrometry of the decrease of NADH by linking with yeast apocarboxylase (EC.4.1.1.1) and alcohol dehydrogenase (EC.1.1.1.1) as follows:



Thiamine + ATP $\xrightarrow{\text{thiaminokinase}}$ thiamine pyrophosphate + AMP

Thiamine pyrophosphate + apocarboxylase $\longrightarrow$ holocarboxylase

Pyruvate $\xrightarrow{\text{holocarboxylase}}$ acetaldehyde + CO_2

Acetaldehyde + NADH + H^+ $\xrightarrow{\text{alcohol dehydrogenase}}$ Ethanol + NAD^+

For studying the effect on pyruvate decarboxylase, 0.2 mμmole of thiamine pyrophosphate was added to the apocarboxylase system with or without the antagonist and the subsequent procedures were the same as those described by Kaziro (12). Both highly purified preparations of pyruvate oxidase complexes and thiaminokinase were prepared by Drs. M. Koike, T. Hayakawa and H. Hamada of the Atomic Disease Institute, Nagasaki.

Compounds. The hydrochloride salt of dimethialium was used in the present studies but the mononitrate salt was used in chick assays. Thiazole-2-^{14}C-labeled thiamine (specific radioactivity, approximately 1 μCi/μmole) and thiamine tetrahydrofurfuryl disulfide labeled with ^{14}C at the corresponding carbon were synthesized by M. Nishikawa and T. Toga of this Division.

RESULTS

Antithiamine action in rat growth. The antithiamine action of dimethialium was clearly demonstrated in both prophylactic and curative assays (table 1 and fig. 1). The growth inhibition induced by the compound was readily reversed by thiamine and the antagonism was of a competitive nature. The antithiamine activity was evident when thiamine was given orally but was no longer appreciable when thiamine was administered parenterally (table 2). However, the administration route of dimethialium did not significantly affect its antithiamine activity, although it declined somewhat when given by intraperitoneal injection. Thiamine propyl

[10] The scintillation fluid consisted of 2,5-diphenyloxazole (PPO), 3g; 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 150 mg; naphthalene, 60 g; dioxane, 1000 ml; and ethyl cellosolve, 200 ml.

[11] TriCarb Model 3000, Packard Instrument Company, Inc., La Grange, Illinois.

[12] Unpublished data.

[13] Unpublished data, H. Tanaka.

disulfide, a thiol-type thiamine derivative, competed with the antagonist three to four times more effectively than thiamine (fig. 2).

Antithiamine action in chick growth. The antithiamine action was also demonstrated in chick growth as judged by incidence of polyneuritis or mortality as well as body weight gain (table 3). Thiamine propyl disulfide counteracted the antithiamine activity of dimethialium (about two times) more effectively than thiamine (group 6 vs. 14, and 7 vs. 15, table 3), as described in the rat experiments. Another series of chick assays showed that dimethialium (25 to 200 mg/kg diet) exerted much less antithiamine activity when thiamine was administered by subcutaneous injection (10 μg/chick/day) (table 4).

Inhibition on thiamine uptake by Ehrlich ascites carcinoma cells. The finding that the antithiamine activity of dimethialium is evident only when thiamine is given orally to rats or chicks, suggests the possibility that the antagonism results from an inhibition of intestinal absorption of thiamine. To affirm this possibility, the effect of the compound on thiamine uptake by Ehrlich ascites carcinoma cells was investigated. First, we confirmed the finding of Menon and Quastel (8) that thiamine was transferred into the cells by an energy-dependent process and most of the intracellular thiamine was characterized as its phosphate esters. We further found that dimethialium inhibited the thiamine uptake (table 5). The inhibition was completely reversed by further addition of thiamine. Therefore, the antagonism was competitive with thiamine and the index was calculated about 25:1 at half inhibition. The thiamine moiety of thiamine tetrahydrofurfuryl disulfide (TTFD), a homologue of thiamine propyl disulfide, was also concentrated into the cells, but much more rapidly and in much higher concentration than thiamine was taken up (fig. 3). The uptake of the disulfide was not inhibited by dimethialium even at the higher ratio of 1000:1. The intracellular thiamine existed largely as free thiamine and any appreciable amount of the disulfide was not detected. The results suggest that, unlike thiamine, thiamine tetrahydrofurfuryl disulfide is transferred into the cells by physical process due to its lipophilic property and then reduced rapidly to thiamine in the cells. The reduction would protect the leakage from the cells and make the process irreversible.

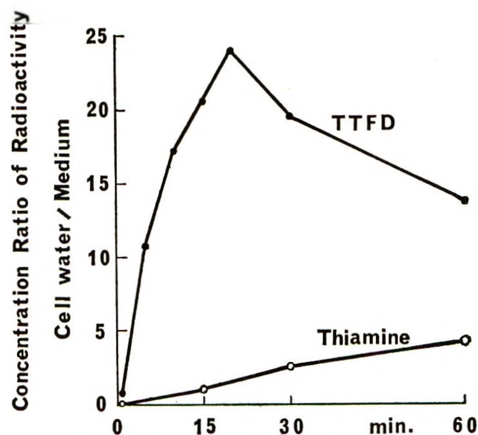


Fig. 3 Uptake of ^{14}C-thiamine or ^{14}C-thiamine tetrahydrofurfuryl disulfide (TTFD) by Ehrlich ascites carcinoma cells.

Effect on thiamine-related enzyme systems. The effects of dimethialium were then examined on 2 types of thiamine-related enzyme systems. The first ones are pyruvate oxidase complexes and decarboxylase which require thiamine pyrophosphate as a cofactor. Dimethialium showed a slight inhibition on the oxidase at a high concentration of 0.01 M (dimethialium: thiamine pyrophosphate, 625:1) (table 6). However, the inhibition appeared not to be responsible for the in vivo antithiamine activity of the antagonist because thiamine itself exerted a similar inhibitory effect. It was also demonstrated that dimethialium did not inhibit decarboxylase even in a high concentration of 4 mM where the molar ratio to thiamine pyrophosphate exceeded 10,000:1 (fig. 4). Therefore, we concluded that dimethialium does not inhibit the cocarboxylase-requiring enzyme systems. The second enzyme studied was thiaminokinase, catalyzing the pyrophosphorylation of thiamine. Dimethialium inhibited the activity and the inhibition was competitively reversed

TABLE 6

Effect of thiamine or dimethialium on pyruvate oxidase complexes

Source of enzyme		Inhibition			
		Pig heart		*E. coli*	
Concn	Molar ratio to TPP [1]	Thiamine	Dimethialium	Thiamine	Dimethialium
mM		%	%	%	%
10	625	17	15	22	30
2	125	3	4	3	7
0.5	25	0	0	0	1

[1] TPP = Thiamine pyrophosphate.

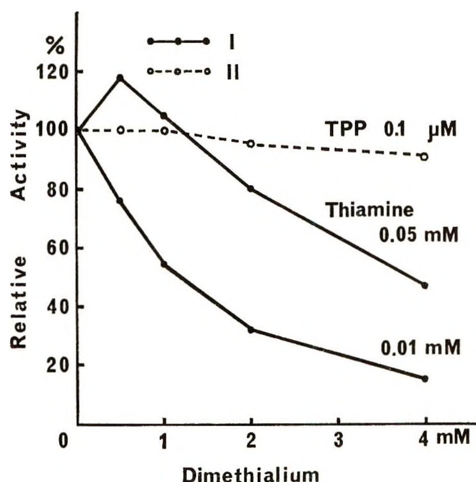


Fig. 4 Effect of dimethialium on thiaminokinase and pyruvate decarboxylase. I, thiaminokinase coupled with apocarboxylase and alcohol dehydrogenase; II, carboxylase coupled with alcohol dehydrogenase. TPP = thiamine pyrophosphate.

by thiamine (fig. 4). The affinity constants were about 6 × 10^{-7} M for thiamine (K_m) and 1 × 10^{-4} M for the antagonist (K_i). Therefore, dimethialium at a molar ratio of 100 to 150 induced one-half inhibition. From these results we concluded that dimethialium is a typical competitive inhibitor for thiamine in the thiaminokinase reaction.

DISCUSSION

The present studies clearly demonstrated the antithiamine action of dimethialium in various biological systems including intact animals, cellular and enzymatic levels. All the observed antagonisms were of a competitive nature with thiamine. Compared with enzymatic studies or microbiological assays, it is difficult to calculate a precise inhibition index from these animal assays. One method is to estimate the ratio of antagonist to the vitamin at which the antagonist counteracts the growth increment induced by a definite amount of vitamin. The ratio can be calculated when the responses are within or close to the linear range of dose-response curve. In the present studies, indexes thus estimated varied from 200 to 1200 for seven independent assays using rats, and from 100 to 300 for 2 assays with chicks. These represent only an approximation of the antithiamine potency of dimethialium, but consistent values are also given by the following observation. In the rat a slight depression in weight gain was noted at a ratio of 100:1 and a severe inhibition was induced with typical deficiency symptoms when the ratio exceeded 1000:1. It seems reasonable, therefore, to assume that the inhibition index is about 500. For the chick the index appears to be approximately 150 since the inhibitory action on chick growth is slight at a ratio of 50:1 but marked at 200:1. The inhibition index of the antagonist in the growth of *L. fermenti* was reported to be 200 to 300 (6) and about 400.[13] These values are of the same magnitude as the values estimated in animal experiments. Comparing our findings with published data on pyrithiamine, oxythiamine and amprolium (5, 13–15), dimethialium is a relatively weak antagonist whose antithiamine activity is far less than that of pyrithiamine but comparable to that of amprolium. Menon and Quastel (8) reported that thiamine was transferred into Ehrlich ascites carcinoma cells by an energy-dependent process and that py-

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rithiamine and amprolium caused one-half inhibition of thiamine uptake at the ratios to thiamine of 2.5:1 and 25:1, respectively. We found that dimethialium inhibited the thiamine uptake competitively and 50% inhibition was induced at a molar ratio of about 25:1. Thus, it is again demonstrated by the in vitro experiment that the antithiamine potency of dimethialium is comparable to that of amprolium and less effective than pyrithiamine.

Pyrithiamine is a potent inhibitor of thiaminokinase ($K_i = 1.3 \times 10^{-7}$ M), and oxythiamine also manifests a substrate competition of this enzyme at a relatively high concentration ($K_i = 1.5 \times 10^{-4}$ M) (1). We have found dimethialium to be a much weaker inhibitor ($K_i = 1 \times 10^{-4}$ M) than pyrithiamine but similar to oxythiamine. We have also determined that dimethialium does not show any significant effect on either pyruvate oxidase or decarboxylase. Eich et al. (16) showed the need for the hydroxyl group in the thiazole ring for antagonistic action of oxythiamine and pointed out the possibility that the active form of the inhibitor might be the pyrophosphate ester. Later, this possibility was confirmed by many investigators who demonstrated the formation of oxythiamine pyrophosphate (1, 17) and its inhibitory effect on decarboxylase systems (2, 16, 18). In this respect, dimethialium does not possess the alcoholic group and therefore it is impossible for the antagonist to be phosphorylated to compete with cocarboxylase.

The finding that dimethialium antagonizes thiamine only when the latter is administered orally to animals is an important feature of its behavior. This suggests the possibility that the primary inhibitory action is on the intestinal absorption of thiamine. That dimethialium blocked the thiamine uptake by Ehrlich ascites carcinoma cells supports this possibility. These findings prompted us to examine the fate of radioactive thiamine given orally to mice or injected into a ligated sac of the small intestine. The results obtained by these autoradiographic studies, to be reported elsewhere in detail, gave experimental evidence supporting the view that the antagonist suppresses the intestinal absorption of thiamine. From all these results, we conclude that the antithiamine activity of dimethialium observed in animal growth results from an interference with intestinal absorption or cellular uptake of thiamine. Of the hitherto known antithiamines, amprolium has been reported to suppress thiamine absorption (19, 20).

Although much evidence has been accumulated for the involvement of the active transport mechanism with cellular uptake of thiamine in mammalian cells (8, 21, 22) and microbial cells (23–26), the underlying mechanism is still unknown. However, these studies suggest that thiamine phosphorylation is a basic process for cellular uptake of thiamine. Hence, an inhibitor of thiaminokinase, such as pyrithiamine or dimethialium, would serve as a useful tool to elucidate the relation between thiamine transport and thiamine phosphorylation. Pyrithiamine inhibited both processes almost equally at a ratio of 5:1 (pyrithiamine/thiamine) (8, 22). We found that dimethialium inhibited thiamine uptake by Ehrlich cells at the ratio of 25:1 where thiaminokinase was not so profoundly affected. Amprolium also blocked thiamine uptake more efficiently than thiamine phosphorylation (8). These facts infer that thiamine transport is more susceptible to these antithiamines than is thiamine phosphorylaton and it is therefore suggested that the latter may not be a sole mechanism involved.

The present studies also show that, unlike thiamine, thiamine tetrahydrofurfuryl disulfide was transferred into Ehrlich cells by a passive process and that dimethialium did not influence the transfer. These results demonstrate that dimethialium inhibits the active transport of thiamine but not the passive uptake of thiamine tetrahydrofurfuryl disulfide. It has been well-established that the disulfide derivatives of thiol-type thiamine, such as thiamine propyl disulfide or thiamine tetrahydrofurfuryl disulfide, are better absorbed from the intestinal tract (27–30) and cause much higher thiamine concentration in blood cells as compared with the administration of thiamine (27). These

characteristics are ascribed to passive transport due to their lipophilic property (31). The present finding that thiamine propyl disulfide counteracts the antithiamine activity of dimethialium two to four times more effectively than thiamine in both rat and chick bioassays could also be explained by the above interpretation that the antagonist does not interfere with the passive transport of the disulfide.

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Effects of Dietary Protein on the Liver of Rats in Experimental Chronic Alcoholism [1,2]

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ABSTRACT The lipotropic effect of dietary protein was tested in male rats fed for 16 weeks a liquid diet in which 40% of the calories were provided by alcohol and 25% by an amino acid mixture. Pair-fed controls received a comparable diet in which sucrose isocalorically replaced the alcohol. The livers were examined by light and electron microscopy and levels of hepatic triglycerides, cholesterol and phospholipids were determined at 4, 8, 12 and 16 weeks. In the early stages (4–8 weeks) of the experiment, the alcohol-consuming rats developed more extensive fatty liver than did controls. The accumulation of fat which was only moderate in degree proved to be transient because it later disappeared (12–16 weeks). But fat in livers of control rats did not disappear. Instead, it progressively accumulated during the entire 16-week period. Some mitochondrial alterations and a few Mallory bodies were also observed at the early stages in rats consuming alcohol. However, these ultrastructural changes sharply regressed so that by the end of the experiment livers from both groups were almost indistinguishable and almost normal (aside from the fat in livers of the carbohydrate controls). These data support the concept that even if alcohol has any postulated hepatotoxic effects on the livers of rats, it can be profoundly modified by manipulation of the accompanying diet.

It has been previously shown by Lieber et al. (1) that rats consuming alcoholic liquid diets containing 16% protein (kcal /100 kcal) for 3 weeks developed moderate hepatic fatty changes and some mitochondrial changes. In our laboratories (2) we have studied the effects of this diet for longer periods (3 months) and were able to confirm these results. In addition we found that the mitochondrial changes had led to the formation of Mallory bodies ("intracellular hyaline").

Lieber has maintained the opinion that the diet was adequate and that the deleterious effects on the liver were therefore attributable to a postulated direct hepatotoxic action of alcohol. However, subsequent publications from his group (3, 4), as well as from ours (5–7),[3,4] indicated that modifications of the original dietary formula, particularly by elevation of the lipotropic content, reduced and even prevented the alcohol-conditioned hepatic changes. It was then obvious that the original liquid diet had, in fact, not been adequate under these special conditions and consequently the hypothesis of a direct toxic effect of alcohol could be seriously questioned.

In the present experiment, we have tested the lipotropic effect of protein in rats that received an alcoholic liquid diet consisting of the following: (in kcal/100 kcal) alcohol, 40; carbohydrates, 20; fat, 15; and protein, 25. The results indicated that even after a period of 16 weeks this diet prevented the accumulation of fat and markedly reduced the extent of ultrastructural changes of the liver. Rather unexpectedly mild degrees of fatty livers developed in the livers of the control rats (high carbohydrate, no alcohol).

MATERIAL AND METHODS

Fifty male rats of the Wistar strain (154–176 g initial body wt) were allotted to 2 groups (A and B) of 25 animals

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[2] This study was supported by the Alcohol and Drug Addiction Research Foundation of Canada.

[3] Gomez-Dumm, C. L. A., and E. A. Porta 1966 Protein and hepatic injury associated with experimental chronic alcoholism. Federation Proc., 25: 304 (abstract).

[4] Porta, E. A., W. S. Hartroft, F. A. de la Iglesia and C. L. A. Gomez-Dumm 1966 Implications of dietary constituents on the lobular distribution of hepatic lesions in experimental chronic alcoholism. Amer. J. Pathol., 48: 39a (abstract).

each. Group A was offered ad libitum an alcoholic liquid diet in which the caloric ingredients (expressed in kcal/100 kcal) were as follows: alcohol,[5] 40; carbohydrate, 20; fat, 15; and protein, 25. The pair-fed controls (group B) received a liquid diet in which sucrose was used to replace isocalorically the alcohol. The composition of the alcohol liquid diet, expressed as grams per 100 ml, was as follows: essential amino acid mixture, 3.050; nonessential amino acids, 3.200; salt mixture (8), 1; vitamin mixture,[6] 2; corn oil, 0.450; olive oil, 1.080; cod liver oil, 0.130; ethanol (absolute laboratory alcohol), 5.714; sucrose, 3; choline chloride, 0.200; sodium carrageenate,[7] 0.400; and distilled water to make 100 ml.

The composition of the essential amino acid mixture in grams/100 g was as follows: L-lysine·HCl 13.434; L-arginine·HCl, 8.125; DL-tryptophan, 2.166; DL-phenylalanine, 9.750; DL-leucine, 17.334; DL-isoleucine, 10.834; DL-valine, 15.167; L-histidine·HCl 5.850; DL-methionine, 6.500; and DL-threonine, 10.834.

Nonessential amino acid mixture, grams/100 g is as follows: L-glutamic acid, 20.639; DL-serine, 5.159; glycine, 7.223; DL-tyrosine, 28.895; L-cystine, 2.063; L-proline, 9.287; L-asparagine monohydrate, 14.344; and DL-alanine, 12.383.

The composition of the isocaloric sucrose diet B was similar to that of diet A, but here the 5.714 g/100 ml of alcohol was replaced by 13 g /100 ml of sucrose.

Both liquid diets provided approximately 1.0 kcal/ml and were offered daily in Richter drinking tubes. No water was given to the animals other than that present in the liquid diets.

To adapt the animals of group A to the high alcohol diet, during the first 4 days they were fed a similar mixture, but with 20% of their calories in the form of alcohol (and sucrose 40%). During the following 3 days a diet with 30% alcohol was offered.

All animals were housed in wire-bottom individual cages in air conditioned rooms supplied during the day with continuous soft music. Body weights were recorded twice weekly.

Five rats from each group were killed at the end of the fourth, eighth, and twelfth week and 10 animals at the end of week 16. The livers were weighed and blocks taken from the median lobes were fixed in Baker's solution (9). Paraffin sections were stained with hematoxylin-eosin, oil red O (10), aniline blue-chromotrope 2R (11) for the detection of Mallory bodies and with Masson's trichrome stain (12) to aid in visualizing the connective tissue. Fat in frozen sections was stained with oil red O (ORO).

Small blocks also taken from the median lobe of each liver were fixed by immersion in Dalton's solution[8] and embedded in Epon 812 (13) for electron microscopy. Thick sections (0.5–1 μ), which were stained with buffered toluidine blue (14), were used for the identification of precise zones of hepatic lobules to be examined by electron microscopy. They were, in addition, stained with a variety of procedures routinely used in our laboratories and reported elsewhere (15) to aid in the visualization of Mallory bodies and when necessary to provide correlation between light and electron microscopy of adjacent sections. Ultrathin sections from preselected lobular areas were stained with lead using Karnovsky's methods (16) and photographed at established initial magnifications. Numbers and surface areas of mitochondria and cells were planimetrically determined by a previously described method (17).

The weighed hepatic remnants from animals killed at the different periods were kept in deep-freeze (− 20°) until biochemical analyses were made. Total hepatic lipids were extracted by the method of Folch et al. (18) and lipid fraction determinations were carried out for triglycerides

[5] Alcohol is used synonymously with ethanol.

[6] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland. The composition of this mixture in mg/100 g is as follows; vitamin A (200,000 IU/g), 450; vitamin D (400,000 units/g), 25; α-tocopherol, 500; ascorbic acid, 4,500; inositol, 500; choline chloride, 7,500; menadione, 225; p-aminobenzoic acid, 500; niacin, 450; riboflavin, 100; pyridoxine·HCl, 100; thiamine·HCl, 100; Ca pantothenate, 300; biotin, 2; folic acid, 9; vitamin B_{12}, 0.135; dextrose to make 100 g.

[7] Viscarin, Marine Colloids Inc., New York.

[8] Dalton, A. J. 1955 A chrome-osmium fixative for electron microscopy. Anat. Rec., *121:* 281 (abstract).

(19), total cholesterol (20) and phospholipids (21).

RESULTS

Food intake. The average daily food intake of rats offered the alcohol diet (group A) and the non-alcohol control diet (group B), expressed in milliliters/100 g body wt, as well as the alcohol intake of group A, expressed in grams per kilogram of body weight, is presented in table 1.

Growth. The amounts consumed were sufficient to promote continued growth and no significant difference was found at any time between body weights of rats given the alcohol diet (group A) and those of pair-fed controls (group B) (fig. 1). The growth rate was 2.69 g/day in group A and 2.67 g/day in group B during the entire experimental period. Animals of group A tolerated the diet well and behaved similarly to those of group B.

TABLE 1

Mean daily food intake

Time	Group	Daily food intake	
		Total	Alcohol
weeks		*ml/100 g body wt*	*g/kg body wt*
4	A	29.70 ± 0.77 [1]	19.48 ± 0.40
	B	29.60 ± 0.40	
8	A	22.60 ± 0.36	14.18 ± 0.24
	B	22.60 ± 0.12	
12	A	20.40 ± 0.80	11.83 ± 0.50
	B	19.70 ± 0.68	
16	A	17.50 ± 0.48	10.24 ± 0.33
	B	17.10 ± 0.23	

[1] Expressed as means ± SE.

Liver weights. No significant differences in liver weights (absolute or per 100 g of body wt) were found between groups A and B at any of the periods studied (table 2).

Triglycerides. Levels of hepatic triglycerides of animals from group A, killed at the end of week 4, were three- to fourfold higher than those considered normal for rats consuming other non-alcoholic liquid diets (2). However, the values in group B were also elevated at this period and not significantly different from those of group A. In group B, the levels remained elevated and did not vary significantly with time. But, in group A, triglycerides reached the maximal peak at week 8 when they were significantly higher than those of group B ($P < 0.05$). However, at the subsequent periods studied (weeks 12 and 16) they returned to normal values and were sig-

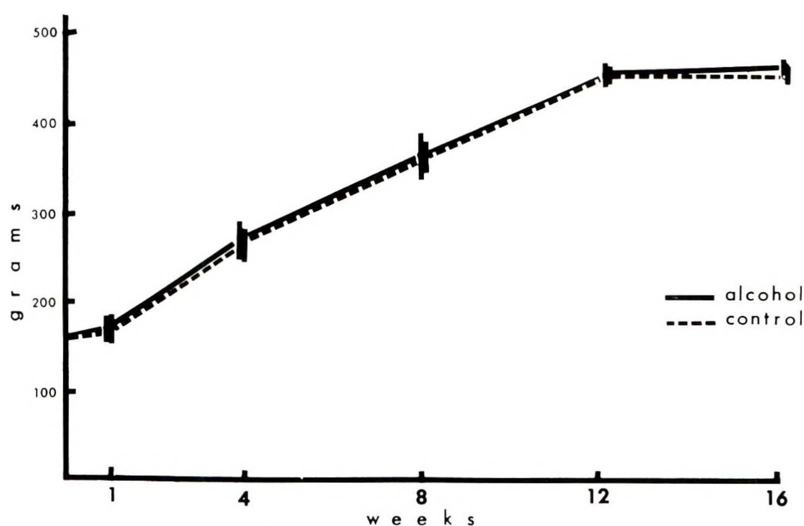

Fig. 1 Changes in body weights of rats consuming the alcohol liquid diet and in pair-fed controls fed a diet in which sucrose replaced the alcohol isocalorically.

TABLE 2

Effects of the diets on the body and hepatic weights and on the hepatic lipids

Time	Group	Body wt	Liver wt	Liver wt	Triglycerides	Cholesterol	Phospholipids
weeks		*g*	*g*	*g/100 g body wt*	*mg/g fresh liver*	*mg/g fresh liver*	*mg/g fresh liver*
4	A	294.28 ± 22.55 [1]	12.49 ± 0.60	4.28 ± 0.19	21.20 ± 3.97	3.00 ± 0.45	17.00 ± 1.95
	B	295.04 ± 19.80	12.21 ± 1.16	4.00 ± 0.43	17.80 ± 4.95	3.20 ± 0.37	12.00 ± 0.71
8	A	379.96 ± 19.88	14.14 ± 0.60	3.74 ± 0.11	37.00 ± 5.58	4.80 ± 0.37	26.20 ± 1.83
	B	377.70 ± 13.36	14.30 ± 0.66	3.78 ± 0.11	16.60 ± 5.86	4.00 ± 0.55	12.20 ± 0.80
12	A	454.20 ± 9.25	16.94 ± 0.31	3.74 ± 0.08	8.80 ± 1.65	2.28 ± 0.34	15.00 ± 1.58
	B	449.00 ± 11.14	15.46 ± 0.75	3.44 ± 0.11	10.80 ± 3.47	2.52 ± 0.45	17.00 ± 1.22
16	A	466.30 ± 6.09	17.55 ± 0.09	3.78 ± 0.07	9.70 ± 2.83	2.68 ± 0.38	16.50 ± 1.12
	B	463.60 ± 7.90	17.52 ± 0.44	3.77 ± 0.07	17.80 ± 1.92	2.92 ± 0.28	28.20 ± 4.37

[1] Expressed as means ± SE.

nificantly lower than those of the control group B ($P < 0.05$).

Cholesterol. Levels of hepatic total cholesterol in groups A and B remained normal and were not significantly different between the groups throughout the experiment.

Phospholipids. Levels of hepatic phospholipids in rats of group A were significantly higher than in those of group B, except at week 12 at which time they were about the same. In both groups the variations with time were somewhat similar to those observed for the triglyceride fraction.

Light microscopy. Sections from livers of group A killed at the end of week 4 showed minimal fatty changes in the form of small droplets which had accumulated in the cytoplasm of centrolobular hepatocytes. By week 8 the amount of stainable fat had sharply increased, but still the changes were confined to centrolobular zones (fig. 2). At this period, the fat droplets were more numerous in affected liver cells but had rarely coalesced to either form globules or displace nuclei. Most centrolobular and some mid-zonal hepatocytes contained variable numbers of Mallory bodies. Their size rarely exceeded those of nuclei. Cytoplasm of some Küpffer cells of centrolobular areas and occasionally those of others contained a few tiny droplets of fat.

By weeks 12 and 16 (fig. 3) stainable fat had almost disappeared from hepatocytes, although most Küpffer cells contained numerous small droplets. Mallory bodies were observed at week 12 but in fewer hepatocytes than at week 8 and only in 3 out of 5 livers of this group. Mallory bodies had completely disappeared in sections from all animals of group A killed at week 16.

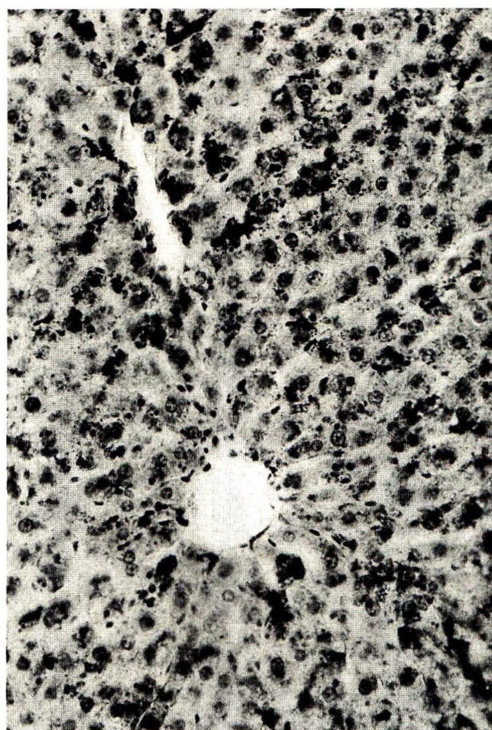

Fig. 2 Accumulation of fat droplets in hepatocytes and Küpffer cells of a rat fed the alcohol liquid diet for 8 weeks. ORO stain. × 600.

Livers from group B showed hepatocytic fatty changes at week 4 which were quantitatively similar to those observed in group

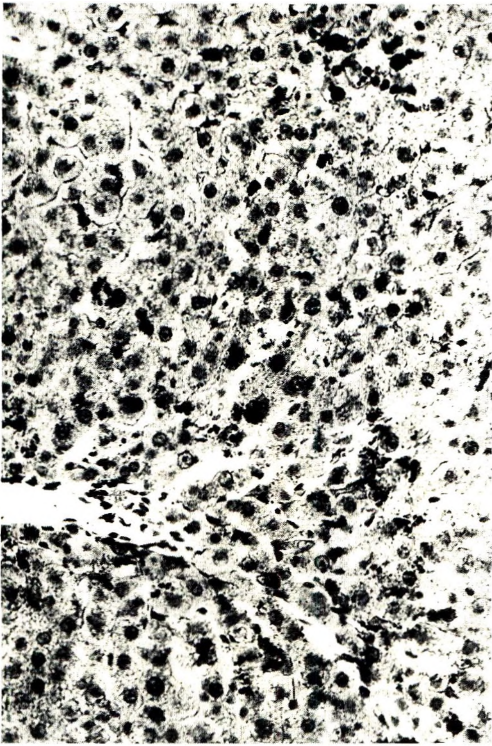

Fig. 3 Fat droplets in Küpffer cells and in some hepatocytes of a rat fed the alcohol liquid diet for 16 weeks. ORO stain. × 600.

A at a similar period; but, the hepatocytes affected were found not only in centrolobular but also in mid-zones. By week 8 (fig. 4) and weeks 12 and 16 (fig. 5), the fatty changes had extended to all areas of the liver lobules and were also found in Küpffer cells. The degree of these changes, however, was not more than moderate and paralleled, in general, the biochemical results.

Electron microscopy. The most noticeable ultrastructural change in hepatocytes of rats of group A killed at week 4 was found in the mitochondria. These organelles were generally enlarged and the configurations of many were bizarre. They had become curved, U-shaped, thin, elongated and even frankly grotesque. Except in those exhibiting very elongated shapes, cristae were scanty, but the density of their matrices and the numbers of opaque bodies did not appreciably differ from normal. The organization of the rough endoplasmic reticulum (RER) varied from cell to cell but slight vesiculization and moderate dislocation of ribosomes was frequent.

By week 8 mitochondrial abnormalities had become even further advanced and the odd-shapes were more commonly found than before (fig. 6). Many megamitochondria were now observed in which only a few peripheral short cristae with variable matrical electron densities could be seen. These megamitochondria corresponded to Mallory bodies as demonstrated in adjacent sections studied by light microscopy and stained by various techniques as reported elsewhere (15). Vesiculation and disorganization of RER were also more profound than at week 4.

At week 12 the mitochondrial changes were similar to those observed at week 8 but the RER showed fewer alterations. By week 16, mitochondria were moderately enlarged but they, as well as the rest of the ultrastructural cytoplasmic elements, had almost normal configuration (fig. 7).

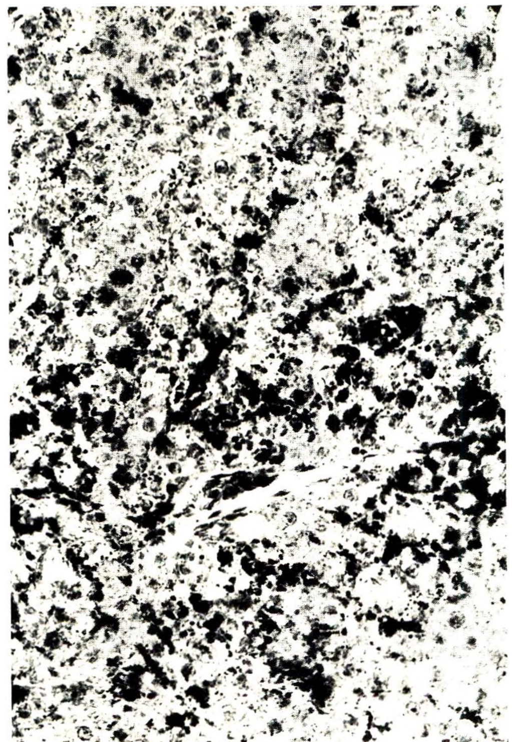

Fig. 4 Fat droplets in hepatocytes and Küpffer cells of a rat fed the non-alcohol diet for 8 weeks. ORO stain. × 600.

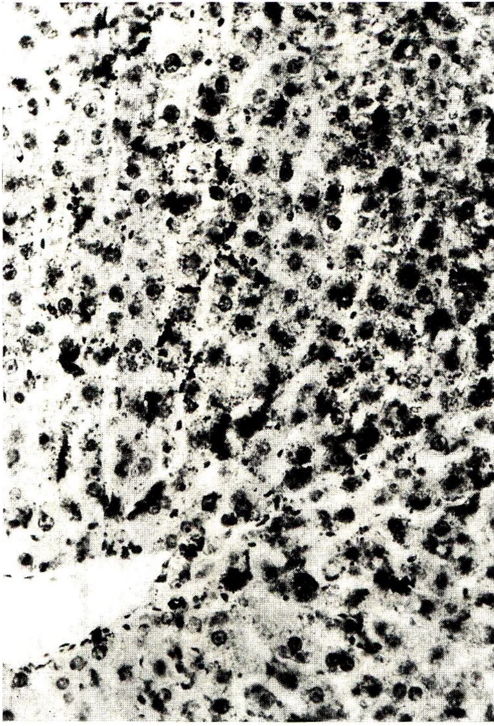

Fig. 5 Accumulation of fat droplets in hepatocytes and Küpffer cells of a rat fed the non-alcohol diet for 16 weeks. ORO stain. × 600.

The Küpffer cells of animals from group A at the different periods studied were ultrastructurally normal except that, with time, increasing numbers of fat droplets appeared in their cytoplasms.

In animals of group B killed at weeks 4, 8 (fig. 8), 12 and 16 (fig. 9), the ultrastructure of hepatocytes was almost completely normal. They contained comparatively more glycogen granules than did those of group A. Küpffer cells in group B showed no appreciable differences when compared with those of group A, also containing small cytoplasmic droplets of fat.

Planimetric determinations (table 3). These determinations showed little difference in the average size of the hepatocytes (area of cells in sections) between groups A and B at the different periods. But, with the exception of weeks 4 and 16, the area of mitochondria per standard (100 μ^2) cell area of group A was almost twice as large as that of group B.

DISCUSSION

Histologic and biochemical results from previous experiments in which rats consumed for 3 to 12 weeks a liquid mixture supplying 36% of alcohol and 16% protein showed that the livers of these animals contained significant amounts of abnormally accumulated fat (1, 2). However, their controls given a similar diet in which the alcohol was replaced isocalorically by sucrose only had moderate amounts of visible fat and slightly elevated hepatic triglycerides. The lipotropic content of these food mixtures was 75 mg (expressed as choline) per 100 kcal, a level that was considered adequate at that time, at least for rats fed conventionally.

It was tempting to conclude then that the alcohol per se and not any dietary anomaly was directly and solely responsible for the fatty changes. However, examination of the proportions of calories from

TABLE 3

Area of cells and number and area of mitochondria per cells in sections

Week	Group	Cell area	No. mitochondria/ cell area	No. mitochondria/ 100 μ^2 of cell area	Area mitochondria/ cell area	Area mitochondria/ 100 μ^2 of cell area
		μ^2			μ^2	μ^2
4	A	487.03	101.16	20.77	147.57	30.30
	B	525.32	98.08	18.29	173.51	33.03
8	A	495.35	263.57	53.21	185.26	37.40
	B	427.80	97.58	22.81	64.72	15.13
12	A	515.20	119.01	23.10	228.18	44.29
	B	538.71	133.11	24.71	139.95	25.98
16	A	501.04	103.71	20.70	139.34	27.81
	B	490.59	133.00	27.11	110.58	22.54

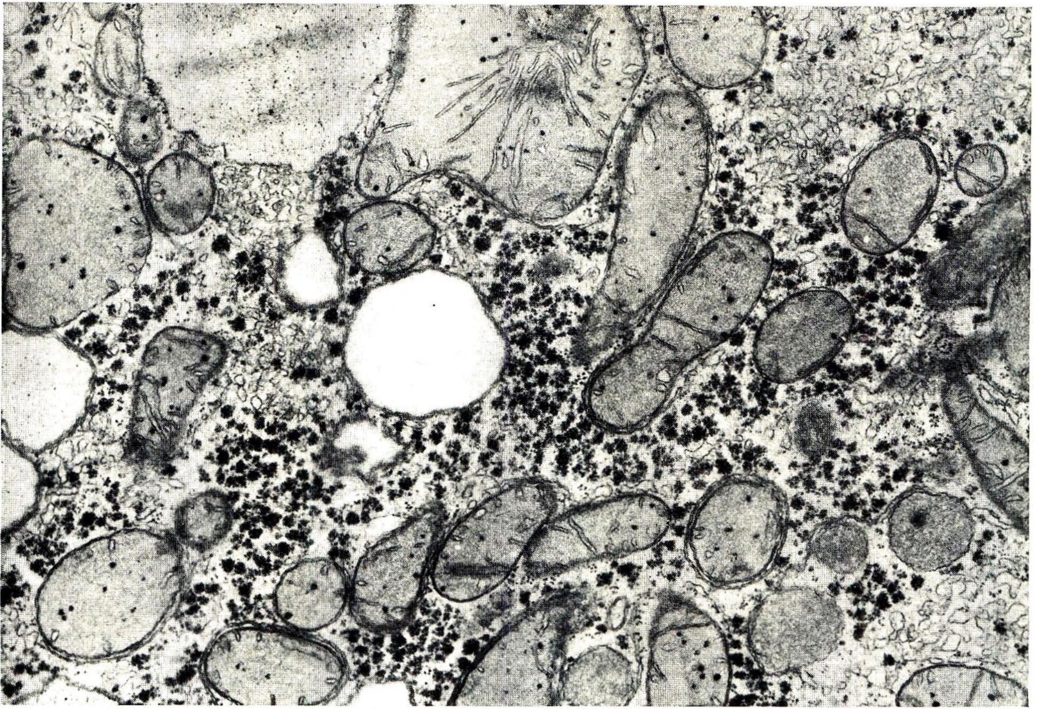

Fig. 6 Mitochondrial alterations in hepatocyte of a rat fed the alcohol liquid diet for 8 weeks. Lead stain. × 20,000.

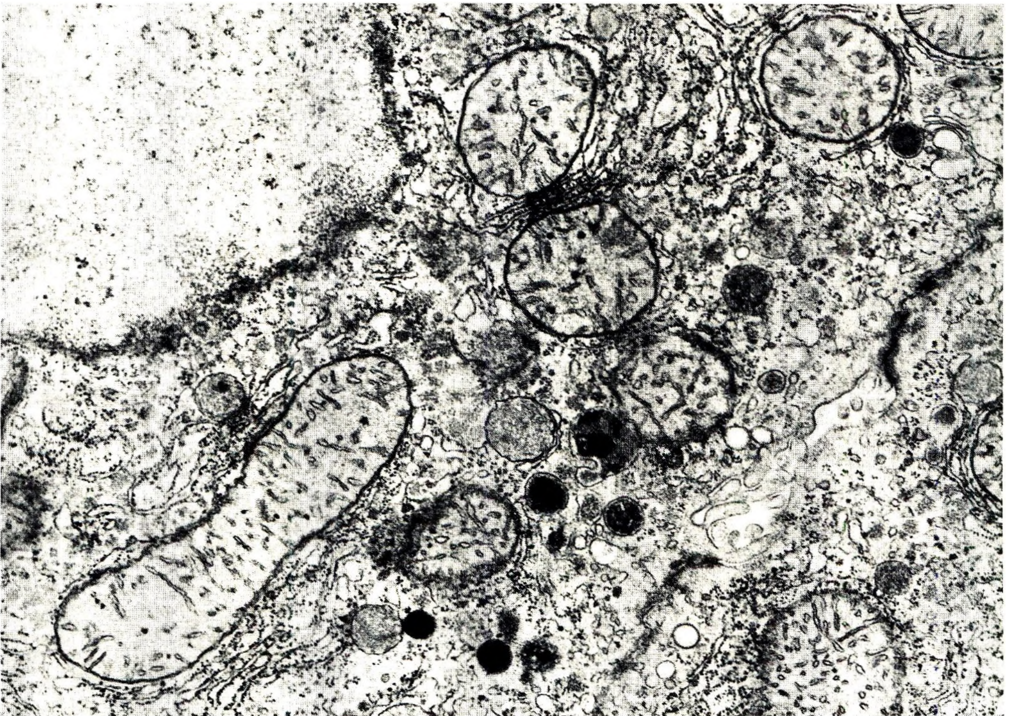

Fig. 7 Aspect of hepatocyte from a rat fed the alcohol diet for 16 weeks. The mitochondria are slightly enlarged but their overall configuration is normal. Lead stain. × 20,000.

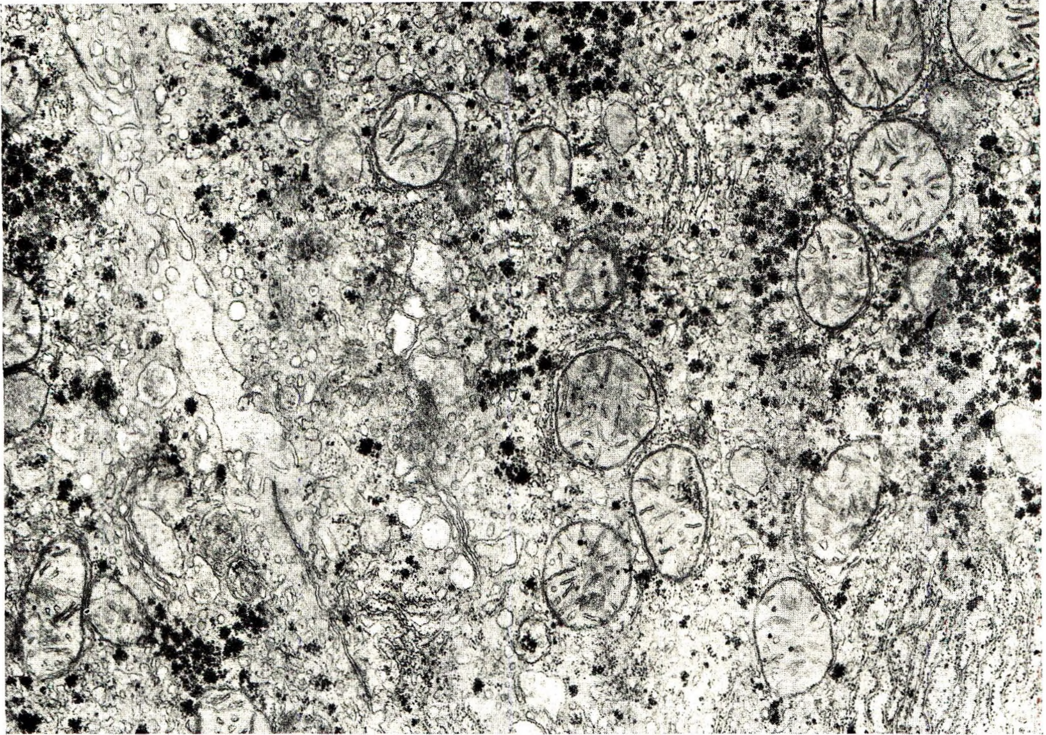

Fig. 8 Normal ultrastructural features in hepatocyte of a rat fed the non-alcohol diet for 8 weeks. Lead stain. × 20,000.

the various food sources showed that this experimental design introduced 2 variables between the control and the experimental animals. The first obviously was the presence of alcohol in one diet and not in the other, and the second (less obvious one) was the uncontrolled imbalance of protein, carbohydrate and fat created by the carbohydrate substitution for alcohol in the control diet. As a result, the percentage of calories from carbohydrate in the alcohol-mixture was only 4 or 5 (coming exclusively from the vehicle in the vitamin mixture), whereas the controls received 41% — over 8 times as much.

The utilization of protein by animals fed low carbohydrate diets has been found to be impaired (22). Therefore the imbalance noted above was at least in theory of relevant importance. Although the ideal relations between nutrients are not yet precisely defined, there is good experimental evidence indicating that the efficiency of food utilization may be seriously affected by alterations in the inter-nutrient balance, as recently emphasized by Crampton (23). These dietary alterations are almost certain to manifest themselves by the development of abnormalities in the liver, as subsequent experiments on chronic alcoholism from our laboratories have shown (24). A diet containing the same caloric proportions of alcohol and protein as before, but with lower amounts of fat (6%) and more carbohydrate (42%), induced significantly less hepatic accumulation of fat than that observed in rats that had consumed a low carbohydrate, high fat mixture. Conversely, other alcohol diets (36%) also low in fat and high in carbohydrate, but with only 6% protein induced severe hepatic alterations. These results taken together demonstrate how markedly the balance of all three chief sources of calories (fat, protein and carbohydrate) can influence results.

In the present experiment the amount of protein was elevated to 25% and that

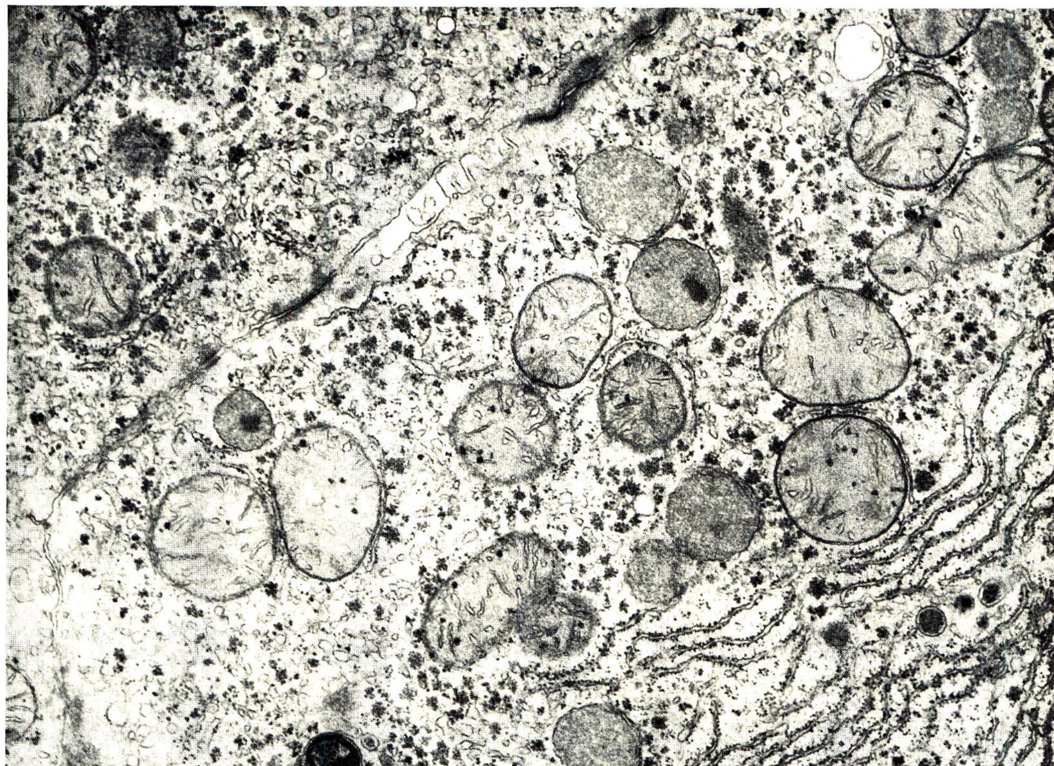

Fig. 9 Normal aspect of hepatocyte from a rat fed the non-alcohol diet for 16 weeks. Lead stain. × 20,000.

of alcohol to 40% of the total calories. Attempts were also made to provide fat and carbohydrate in more harmonious proportions under these circumstances (20% carbohydrate versus 5% Lieber's experiment). The lipotropic content of this diet, as well as that consumed by the controls in which alcohol was isocalorically replaced by sucrose was high (269 mg/100 kcal). The results showed that in the earlier stages of the experiment (4–8 weeks) the alcohol-consuming rats developed more extensive fatty liver than controls, but this finding proved to be a transient one because it disappeared after longer periods (12–16 weeks). However, the control animals developed more extensive fatty liver at the end of the experiment than did the alcohol-rats. We did not expect to find fatty liver (albeit to a slight degree: 17.80 mg of triglycerides/g of wet liver) in the rats of control group B at the end of the experiment because the ratio of carbohydrate, fat and protein (60:15:25) is comparable to that which many other investigators and ourselves have often used in conventional solid food mixtures in" normal" diets for rats. The differences between the control diet in the present experiment and the conventional ones are that the rats consumed the mixture in a liquid form and protein had been replaced by amino acids. Further studies are necessary to seek the explanation.

The protective effect of these relatively high levels of protein (25%) has also been demonstrated by Jones and Greene (3) who showed that the livers of rats were protected if the animals consumed liquid diets providing 36% of the calories as alcohol, as long as the rest of the food mixture was relatively low in fat (5%) and high in protein (19%).

These data strongly support the concept that any postulated hepatotoxic effects of alcohol on the livers of experi-

mental animals can be vastly modified by manipulation of the accompanying diet, lipotropic content and particularly the level of protein. It is difficult for us, in the light of these data, to ascribe to alcohol a "direct hepatotoxic" action.

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Thiamine Monophosphate, a Normal Constituent of Rat Plasma

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ABSTRACT In plasma of normal rats the presence of thiamine monophosphate, in a completely dialyzable form, was demonstrated by chromatographic procedures. Its content (16.2 ± 0.47 µg/100 ml) accounted for 64% of plasma total thiamine, the rest being free thiamine. Both thiamine compounds of plasma were rapidly and greatly affected by dietary thiamine deficiency. Administration of thiamine or its monophosphate by various routes apparently modified only the free thiamine content. Phosphorylated thiamine (most likely monophosphate) was present in the plasma of all the animal species studied.

Some of our preliminary experiments showed that incubation of rat blood plasma with human prostate phosphatase caused a net increase of free thiamine. This increase could have been a consequence of the hydrolysis of some thiamine phosphates present in plasma. Since there is little or no information on the nature and physiological meaning of thiamine phosphates in plasma, we conducted some experiments to clarify these points; the results are presented here briefly.

MATERIAL AND METHODS

Male albino rats (Wistar strain), 150 to 180 g starting weight, were maintained with a complete purified diet.[2]

Unless otherwise stated, the blood was withdrawn from the abdominal aorta of laparotomized rats under light anesthesia by a heparinized syringe. Plasma was separated by centrifuging for 10 minutes at 2500 rpm.

Thiamine was determined by the method of Rindi and Perri (1) with minor modifications. The fluorescence was measured in a Beckman DU (model G 2400) spectrophotometer, equipped with fluorimetric apparatus. Thiamine and its esters were extracted from blood as follows: to 2 ml of plasma (or 0.5 ml of whole blood) were added 4 ml of distilled water, followed by 0.8 ml of 30% trichloroacetic acid (TCA) (w/v). After 10 minutes, the sample was centrifuged and the precipitated proteins, thoroughly suspended in 2.5 ml of 2% TCA, were again centrifuged. The combined supernatant fluids were brought to pH 5.1 with 1.2 ml of 2.5 M sodium acetate and diluted with distilled water to 10 ml in a volumetric flask. One milligram of human prostate phosphatase, prepared according to Vescia and Testi (2), lyophylized, and dissolved in 0.1 ml of water, was added and the solution incubated at 37° overnight. A second aliquot of 2 ml of the same plasma, similarly treated, was incubated without phosphatase. In both samples thiamine was fluorimetrically determined, the first yielding the total thiamine and the second the free thiamine content. Phosphorylated thiamine was calculated by difference (2). When ethylenediaminetetraacetic acid (EDTA) was present, 25 mg of $CaCl_2$ were added before incubation to eliminate its inhibiting action on prostate phosphatase.

The resins used were: Dowex [3] 1-X8, (2000–400 mesh), acetate (3); Amberlite [4] XE 64, both carboxylic or buffered at pH 6.5 (4).

The thiamine (chloride hydrochloride), thiamine monophosphate (chloride) and thiamine diphosphate were commercial products.[5]

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[1] Present address: Vister Research Laboratories, Casatenovo Bz., Italy.

[2] Obtained from S.p.a.F. Vismara, Casatenovo Bz. (Como), Italy.

[3] Dowex, Dow Chemical Company, Midland, Michigan.

[4] Amberlite, Rohm and Haas Company, Philadelphia, Pennsylvania.

[5] Kindly supplied by Prodotti Roche, Milan.

All the chromatographic columns were made of Pyrex glass tubes, fitted with a sintered glass disk and fused to a ground-glass stopcock.

EXPERIMENTS AND RESULTS

Experiment 1. Total thiamine content of rat whole blood and plasma. The results of several determinations were: (in μg/100 ml ± SE) total thiamine of whole blood, 28.31 ± 0.75 (7 rats) and of plasma, 25.25 ± 0.47 (12 rats), which show that the plasma total thiamine content did not greatly differ from that of the red cells.

Experiment 2. Free and total thiamine content of blood plasma. The mean values for plasma samples from 8 rats were: (μg/100 ml ± SE) total thiamine, 25.25 ± 0.47; and free thiamine, 9.05 ± 0.32. Incubation with phosphatase, necessary for total thiamine determination, caused an increase of free thiamine, probably by hydrolyzing thiamine phosphates, which amounted to 64% of the total thiamine.

Experiment 3. Incubation of plasma with or without addition of thiamine phosphates. The well-known plasma phosphatase activity might easily split thiamine phosphates, either added or preformed. This possibility was investigated by incubating several 3-ml samples of rat plasma at 37°, soon after their withdrawal from the aorta, for different periods of time and determinining their free and total thiamine content.

Figure 1 shows that incubation increased free thiamine in plasma, did not modify total thiamine, and lowered esterified thiamine. Further evidence of intrinsic plasma phosphatase activity came from experiments in which a set of blood plasma samples (2 ml) was incubated at 37° after addition of 0.8 μg of thiamine diphosphate. The enzymatic activity was interrupted at different times by adding 30% TCA solution. After centrifuging and washing as described above, in the supernatant fluid only free thiamine was determined. The results (fig. 2) again suggest that rat plasma can hydrolyze thiamine phosphates enzymatically.

Experiment 4. Blocking of plasma phosphatase activity. A reliable determination of plasma thiamine phosphates could be made only after inhibition of phosphatase activity, which otherwise could destroy them. The following experiments show the efficacy of EDTA as a plasma phosphatase inhibitor.

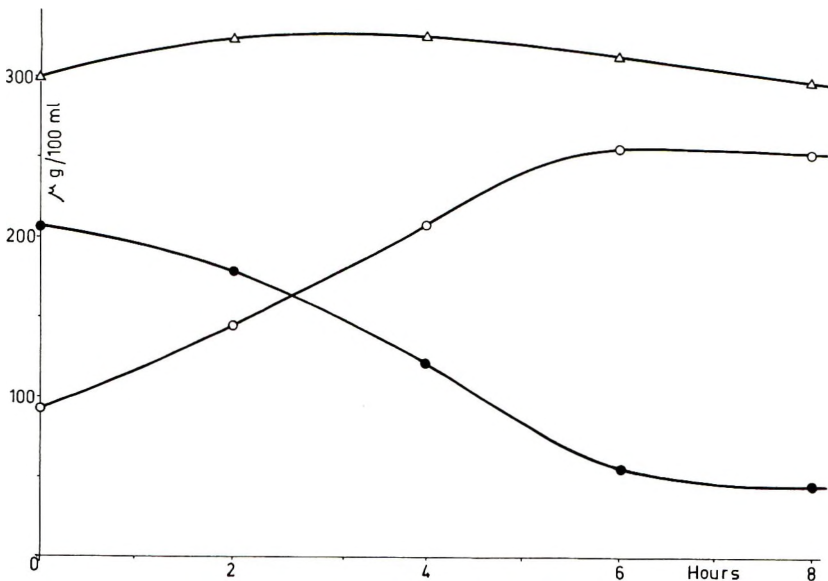

Fig. 1 Influence of incubation at 37° on rat plasma content of: –○–, free thiamine; –●–, phosphorylated thiamine; –△–, total thiamine.

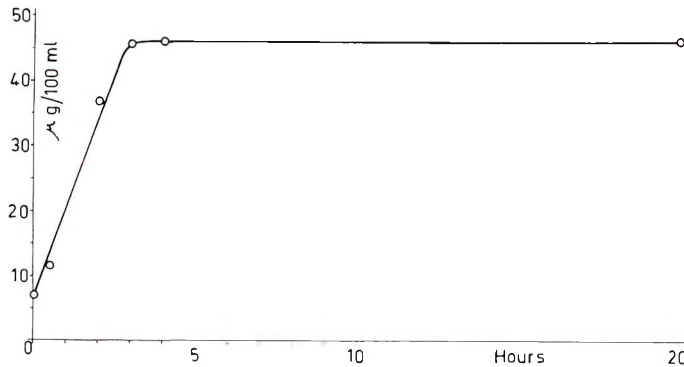

Fig. 2 Free thiamine content of rat plasma incubated at 37° with thiamine diphosphate (0.4 µg/ml); each plasma sample was 2 ml.

To blood, withdrawn using a syringe rinsed with 5% heparin solution containing 1% NaCl and 2.5% EDTA, 1 mg of EDTA per ml was added and mixed thoroughly. From the plasma several 2-ml samples were prepared, to some of which 0.3 µg of thiamine mono- or diphosphate, dissolved in 0.1 ml of saline, was added. The free and total thiamine content was determined on all samples, before and after five hours of incubation at 37°. The lack of an increase in free thiamine (table 1) after incubation clearly shows the efficacy of EDTA as an inhibitor.

Experiment 5. Identification of thiamine monophosphate in plasma. Different chromatographic procedures were used to identify the thiamine phosphates normally present in plasma.

Seventy-five milliliters of rat plasma, collected in the presence of EDTA, were diluted with 75 ml of water and deproteinized with 37 ml of 30% TCA solution, mixing thoroughly. After 10 minutes the proteins were centrifuged, washed with 20 ml of 2% TCA and again centrifuged. The supernatant fluids were combined, brought to pH 6.8 to 7.0 with 10% NaOH and passed through a column (1.3 cm × 8 cm) of deactivated charcoal, prepared according to Rindi and de Guiseppe (3). After washing with 20 ml of water, thiamine and thiamine phosphates were eluted with 80 to 100 ml of 10% *n*-propanol in water.

The eluate was concentrated to 20 ml in a rotating evaporator under vacuum, at 40°. An aliquot was used for the determination of free and total thiamine content and the rest was subjected to the following analytical procedures.

A. Six milliliters of the concentrated propanolic eluate were acidified with 0.8 ml of 0.1 N HCl and placed on a chromatographic column (0.8 cm × 3 cm) of Dowex 1–X8, acetate. Separate determinations of thiamine, thiamine mono- and diphosphate were performed according to Rindi and de Giuseppe (3). Two fractions were eluted: the first (fraction I) contained thiamine plus thiamine monophos-

TABLE 1

Inhibition of rat plasma phosphatase activity by EDTA [1] *(exp. 4)*

Incubation	Plasma		Plasma [2] + thiamine monophosphate		Plasma + thiamine diphosphate	
	Free thiamine	Total thiamine	Free thiamine	Total thiamine	Free thiamine	Total thiamine
hours	*µg/100 ml*		*µg/100 ml*		*µg/100 ml*	
0	5.12	16.02	5.12	41.60	5.12	40.30
5	5.10	16.04	5.02	39.80	5.15	39.80

[1] Averages of 2 experiments.
[2] 0.3 µg/ml of thiamine mono- or diphosphate were added to plasma before incubation.

phate and the second (fraction II), thiamine diphosphate.

B. Six milliliters of the same concentrated propanolic eluate were passed through a column (0.8 cm × 3 cm) of Amberlite XE 64 resin, buffered at pH 6.5, which retained free thiamine and allowed phosphorylated thiamine (thiamine mono- and diphosphate and other esters) to flow through. Thiamine phosphates were collected by washing with 20 ml of water (fraction I): the successive fraction (fraction II), eluted with 20 ml of 0.5 N HCl contained all the free thiamine.

C. Finally, 6 ml of the concentrated propanolic eluate were passed through a column (0.8 cm × 3 cm) of Amberlite XE 64 resin, carboxylic, which retained both free and phosphorylated thiamine (thiamine mono- and diphosphate). After rinsing with 25 ml of water (fraction I), all thiamine compounds previously retained by the resin were eluted with 20 ml of 0.5 N HCl (fraction II).

On each fraction separated in the single chromatographic procedures described, the free and total thiamine content was determined, phosphorylated thiamine being calculated as the difference.

In table 2 the results of a typical experiment are summarized. All the chromatographic procedures assured good recovery of both free and phosphorylated thiamine of the original plasma sample. Moreover, procedure A showed that the only thiamine phosphate in plasma was thiamine monophosphate. This was qualitatively confirmed by paper chromatography. Each fraction eluted in single chromatographic procedures (see table 2) was concentrated to 0.1 ml in a rotating evaporator and chromatographed on OB Muntkell paper, according to Siliprandi and Siliprandi (5), for 12 hours. However, no clear separation could be obtained for thiamine compounds contained in fractions of procedures A and B, owing to the presence of many interfering substances. Only the fraction eluted with HCl in procedure C was completely free of impurities. In the experiment referred to in figure 3 such a fraction was eluted with 0.01 N HCl to avoid strong acidification during the concentration of the sample. The paper chromatogram (fig. 3) shows clearly that thiamine in rat plasma was present only in 2 forms, free and monophosphate.

Experiment 6. Dialysis of plasma. The possibility of thiamine monophosphate being bound to plasma proteins was tested with dialysis experiments. Twenty-two milliliters of rat plasma, collected in the presence of EDTA, were dialyzed in Visking tubing,[6] against 2 liters of 0.015 M phosphate buffer pH 7.2 (containing 1 mg EDTA/ml), at 4° for 24 hours with slow agitation. Free and phosphorylated thiamine content of plasma, before and after dialysis, as well as of the dialysis fluid, con-

[6] Visking Company, Chicago.

TABLE 2

Composition of fractions obtained by different chromatographic procedures [1] (exp. 5)

Chromatographic procedure	Fraction	Eluents	Thiamine compounds present in fractions as:		Content found in fractions	
			Free thiamine	Phosphorylated thiamine	Free thiamine	Phosphorylated thiamine
					µg/100 ml plasma	
None (plasma)	—		thiamine	TMP [2] + TDP	4.82	15.40
A Dowex 1-X8 acetate	I	H_2O	thiamine	TMP	4.68	14.91
	II	0.1 N acetic acid	—	TDP	0	tr
B Amberlite XE 64, buffered (pH 6.5)	I	H_2O	—	TMP + TDP	tr	13.20
	II	0.5 N HCl	thiamine	—	4.52	tr
C Amberlite XE 64, carboxylic	I	H_2O	—	—	0	0
	II	0.5 N HCl	thiamine	TMP + TDP	4.97	13.40

[1] The results of a typical experiment are reported.
[2] TMP, TDP = thiamine mono-, diphosphate.

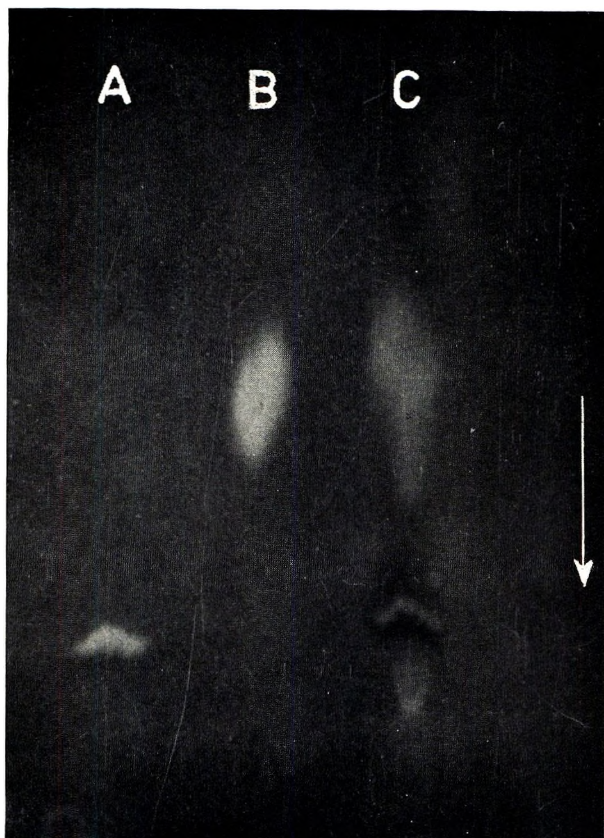

Fig. 3 Paper chromatogram of fraction II of purified rat plasma extract. The chromatographic solvent was *n*-propanol:water:M acetate buffer, pH 5 (70:20:10). Elution from Amberlite XE 64 resin, carboxylic, was made with 0.01 N HCl (procedure C; see text). Fluorescence was developed by spraying with alkaline ferricyanide. *A*, thiamine; *B*, thiamine monophosphate; and *C*, fraction II.

centrated in the vacuum, was determined. Practically all the plasma thiamine monophosphate was dialyzed (table 3); this means that it was not in a protein-bound form. Also free thiamine was dialyzable.

Experiment 7. Free and phosphorylated thiamine in plasma of different animals. All the determinations were made on plasma samples collected in the presence of EDTA. The results reported in table 4 show that phosphorylated thiamine (probably thiamine monophosphate) was found in plasma of all the animals we studied. In dogs, the amounts of thiamine monophosphate and free thiamine were so small that they could not be detected with certainty by the method we used. In mammals the amount of thiamine monophosphate was rarely smaller that that of free thiamine.

TABLE 3

Effect of dialysis on plasma thiamine (free and phosphorylated) [1] *(exp. 6)*

Material	Free thiamine	Phosphorylated thiamine
	μg	μg
Plasma	1.40	4.23
Plasma after dialysis	0.20	0.40
Dialysis fluid	1.28	3.48

[1] Averages of 2 experiments.

Experiment 8. Effect of dietary thiamine deficiency on TMP plasma content. Male albino rats (Wistar strain), weighing about 70 g, were kept in individual cages and fed a thiamine-deficient diet consist-

TABLE 4

Free and phosphorylated thiamine content of plasma of various animals (exp. 7)

	No. determinations	Free thiamine	Phosphorylated thiamine
		μg/100 ml	*μg/100 ml*
Rabbit	3	4.00 ± 0.12 [1]	3.91 ± 0.71
Guinea pig	3	1.21 ± 0.21	1.56 ± 1.56
Hog	3	1.52 ± 0.04	3.30 ± 0.12
Rat	7	9.37 ± 0.32	15.61 ± 0.70
Dog	2	tr	tr
Turkey	2	3.40 ± 0.60	0.20 ± 0.01
Chick	4	2.86 ± 0.84	0.38 ± 0.06

[1] Mean ± SE.

TABLE 5

Plasma rat thiamine and thiamine monophosphate content during dietary thiamine deficiency (exp. 8)

Days of deficiency	No. determinations	Free thiamine	Thiamine monophosphate
		μg/100 ml	*μg/100 ml*
0	8	9.05 ± 0.32 [1]	16.20 ± 0.71
2	5 [2]	4.60 ± 0.25	3.60 ± 0.44
4	3 [3]	1.49 ± 0.11	1.49 ± 0.11
6	2 [3]	0.57 ± 0.06	0.64 ± 0.07
8	2 [3]	0.51 ± 0.02	0.38 ± 0.08
10	2 [3]	0.38 ± 0.02	0.26 ± 0.02

[1] Mean ± SE.
[2] Each value was obtained on plasma pooled from 4 rats.
[3] Each value was obtained on plasma pooled from 10 to 15 rats.

ing of: (in percent) casein, washed and defatted, 18; cornstarch, 65; olive oil, 2; and Osborne and Mendel salt mixture (6), 5. The diet was supplemented with the following amounts of vitamin B complex, excluding thiamine, given by mouth on alternate days: (in μg) riboflavin 80; pyridoxine, 40; nicotinamide, 800; pantothenate, 4; *p*-aminobenzoic acid, 20, inositol, 40; choline, 850. In addition, every week 1 mg of *dl*-α-tocopherol was administered per os. The course of thiamine-deficiency, as indicated by thiamine content of the tissues, was previously studied by De Caro et al. (7).

Groups of rats were decapitated at various times and free and thiamine monophosphate content were determined on 2-ml samples of plasma separated from the pooled blood in the presence of EDTA.

The results (table 5) show that thiamine deficiency affected both free thiamine and its monophosphate plasma content considerably: in 10 days they were reduced to extremely low values. The time course of the decrease, although similar, is quantitatively different for the 2 compounds, being much sharper for thiamine monophosphate, particularly in the first 4 days of deficiency. Thereafter the differences decreased until only traces of both compounds remained in plasma.

Experiment 9. Effect of administration of thiamine monophosphate or thiamine. Two groups of normal male albino rats of 150 g body weight received intravenous injections (tail vein) of the following thiamine compounds, dissolved in 0.2 ml of saline: group A, 350 μg of thiamine; and group B, 450 μg of thiamine monophosphate 20. A third group (group C) received 1.05 mg of thiamine per os. After different time-intervals, blood was collected and pooled from 2 animals in the presence of EDTA. The free and total thiamine content was determined on the plasma.

The results of this experiment (table 6) show that for all 3 groups of rats the greatest increase was in free thiamine content, irrespective of the type of compound administered; this means that thiamine monophosphate was rapidly hydrolyzed. In group A, 5 minutes after the intravenous thiamine injection the

TABLE 6

Effect of thiamine or thiamine monophosphate (TMP) administration on thiamine and TMP content of rat plasma (exp. 9)

Compound injected and route	Time after administration	Free thiamine	Thiamine monophosphate
	min	*μg/100 ml*	*μg/100 ml*
	Group A		
350 μg thiamine, iv	0	9.05 ± 0.32 [1]	16.20 ± 0.71
	5	229.00 ± 18.00	3.03 ± 1.00
	20	47.00 ± 8.00	22.02 ± 0.70
	60	16.00 ± 0.20	16.10 ± 0.10
	120	9.80 ± 0.20	15.20 ± 0.20
	Group B		
450 μg TMP, iv	0	9.05 ± 0.32	16.20 ± 0.71
	5	230.00 ± 20.00	186.00 ± 12.00
	20	48.00 ± 3.00	16.00 ± 3.00
	60	24.00 ± 0.40	28.00 ± 4.00
	120	11.00 ± 0.20	22.00 ± 0.10
	Group C		
1.05 mg thiamine, per os	0	9.05 ± 0.32	16.20 ± 0.71
	5	10.50 ± 0.30	21.00 ± 1.30
	20	15.05 ± 0.20	20.00 ± 1.20
	60	12.50 ± 0.30	20.60 ± 0.10
	120	10.45 ± 0.40	22.05 ± 0.50

[1] Mean ± SE of 4 different determinations, each made on plasma pooled from 2 animals.

thiamine monophosphate content appeared to disappear completely. This was certainly an artifact due to the method of determination we used, whereby the thiamine monophosphate was taken as the difference between total and free thiamine. The latter was markedly increased by the intravenous injection, hence rendering the thiamine monophosphate determination extremely uncertain. Only in group B, 5 minutes after the intravenous injection was the thiamine monophosphate content clearly but temporarily increased.

DISCUSSION AND CONCLUSIONS

The results of our experiments indicate clearly that thiamine monophosphate is present in the normal plasma of various animals, particularly in rats. The use of EDTA in the collection of the plasma samples prevented the splitting of thiamine monophosphate as well as its new formation from higher thiamine phosphates, as Kiessling and Tilander (8, 9) showed in liver. As dialysis indicated, plasma thiamine monophosphate is completely diffusible.

None of the experimental conditions we tested (dietary thiamine deficiency, administration by different routes of thiamine or thiamine monophosphate) specifically affected the thiamine monophosphate plasma content. However, a finding that must be emphasized is that plasma thiamine monophosphate appeared to be extremely sensitive, particularly in the first few days of thiamine deficiency, to the lack of thiamine in the diet.

As to the origin of plasma thiamine monophosphate, it probably comes from several tissues. In fact thiamine monophosphate has been found, although in small amounts, in various organs (brain, heart, liver, kidney) of many animals (10) as well as in peripheral nerves (10).[7] As in plasma, the organ content of thiamine monophosphate is not affected by thiamine dietary deficiency (7) or thiamine and thiamine monophosphate administration (11). Recently Gregory and Kon (12) were able to detect thiamine monophosphate in milk of various animals, confirming a previous finding of de Jong (13).

Probably thiamine monophosphate is a catabolic product of thiamine diphosphate,

[7] Gurtner, H. P. 1957 Beitrag zur frage des Aneurinstoffwechsels während der Nervenerregung. Helv. Physiol. Pharmacol. Acta, 15: C 66.

the major thiamine compound of tissues (10) or of thiamine triphosphate. It has no enzymatic activity, at least in those enzymatic systems in which thiamine diphosphate is active and it is not an intermediary product in the synthesis of thiamine diphosphate by animal tissues (14).

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Dialysis Studies of Liver Zinc in Zinc-deficient and Control Rats [1]

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ABSTRACT Experiments were designed to study possible effects of zinc deficiency on zinc binding by rat liver. Either 3 hours after intravenous ^{65}Zn injection or after 10 days of feeding ^{65}Zn-containing diets, low zinc (−Zn) and zinc-supplemented (+Zn) rats were killed and liver homogenates dialyzed against 8 successive volumes of buffered 10^{-3} M EDTA, CDTA, histidine, cysteine, glycine, or buffer alone. Based on the dialysis results, liver zinc was delineated into 4 pools. When ^{65}Zn was fed for 10 days to label zinc pools uniformly, dietary zinc deficiency had no significant effect on the amount of ^{65}Zn removed by any of the dialyzing agents, which suggests that the binding pattern of liver zinc, like that of total zinc concentration, is not altered in zinc deficiency. When ^{65}Zn was injected intravenously, the percentage of liver ^{65}Zn removable at 3 hours postinjection by EDTA, but not by histidine, was significantly smaller in −Zn than in +Zn rats. This suggests that the rate of incorporation of zinc into certain liver pools is altered in zinc deficiency. Explanations for this effect were suggested, based on the difference in growth rate between the +Zn and −Zn rats and on the possible increase in the turnover rate of a firmly bound zinc pool in zinc deficiency.

The zinc concentration of plants and microorganisms grown under zinc-deficient conditions is often greatly reduced in comparison with control values (1–5). In contrast, most animal tissues show little or no decrease in zinc concentration due to dietary zinc deficiency, even when growth is severely impaired. In the rat, the only organs in which consistent and significant decreases in zinc concentration have been observed in zinc deficiency are the dorsolateral prostate, testes, and epididymis (6–8) and bone (7–9). This suggests that most animal tissues must monitor something other than the total zinc concentration when the supply of available zinc is not sufficient to permit continued cell division and growth. The experiments reported here were designed to study possible effects of zinc deficiency in rats on certain parts of the total pool of liver zinc, as delineated by dialysis. The radioisotope ^{65}Zn was used to label liver zinc pools. The results indicate that the liver zinc pools, delineated by dialyzing agents of varying zinc affinities, are not significantly altered in size due to zinc deficiency, but that the rate of incorporation of zinc into certain of these pools is affected by the deficiency.

MATERIALS AND METHODS

Weanling male rats of the Holtzman strain were fed either the basal, low zinc diets shown in table 1, or the same diets supplemented with 20 ppm zinc as zinc carbonate. The zinc content of the basal diet was routinely in the range of 0.8 to 1.2 ppm zinc, as determined by atomic absorption spectrophotometry.[2] As indicated in table 1, the amino acid source of the basal diet was changed from acid-hydrolyzed casein to spray-dried egg white between experiments 2 and 3 for economy. Rats fed the low zinc (−Zn) diet were housed individually in stainless steel cages; those fed the zinc-supplemented (+Zn) diet were housed individually in galvanized cages. Distilled water in glass bottles and feed in porcelain cups were fed ad libitum.

The onset of zinc deficiency occurred at about 8 to 12 days, as determined by re-

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[2] Model 214 Atomic Absorption Spectrophotometer, manufactured by the Perkin-Elmer Company, Norwalk, Connecticut.

TABLE 1

Composition of the basal diets

	Exps. 1 and 2	Exps. 3 and 4
	g/kg	*g/kg*
Glucose monohydrate [1]	633.7	635.9
Acid-hydrolyzed casein, salt-free [2]	200.0	—
L-Tryptophan [2]	2.2	—
Spray-dried egg white [2]	—	200.0
Cellulose [3]	60.0	60.0
Corn oil	55.0	55.0
Mineral mixture [4]	45.1	45.1
Vitamin mixture [5]	2.5	2.5
Choline chloride	1.5	1.5

[1] Cerelose, Corn Products Company, New York.
[2] General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] Solka Floc, Brown Company, New York.
[4] Provided in g/kg of diet: $CaCO_3$, 12.3; $CaHPO_4$, 0.267; KH_2PO_4, 17.1; NaCl, 12.5; $MgSO_4$, 2.425; $Fe(C_6H_5O_7)$, 0.149; $MnSO_4 \cdot H_2O$, 0.058; $CuSO_4$, 0.058; and in mg/kg of diet: KI, 0.249; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.175.
[5] Provided in mg/kg of diet: *dl-a*-tocopheryl acetate, 400; inositol, 100; ascorbic acid, 50; nicotinamide, 25; Ca pantothenate, 20; thiamine, 5; riboflavin, 5; pyridoxine·HCl, 2.5; menadione, 0.5; folic acid, 0.2; biotin, 0.1; cyanocobalamin, 0.1% triturate, 0.02; and in IU/kg of diet: vitamin D_2, water-dispersible, 10,000; and vtiamin A palmitate, gelatin-coated, 5,000. The egg white-containing diet was supplemented with additional biotin at the level 1 mg/kg of diet.

duced growth and the appearance of one of more of the following symptoms: redness and cracking of the tail and paws, dermatitis, and loss of hair. Thereafter, growth was rapidly and severely depressed, as indicated in table 2.

When growth of the −Zn rats had been severely impaired, a tracer dose of ^{65}Zn (8–10 μCi/rat) was administered as the zinc-glycine complex prepared as described by Heth and Hoekstra (10). In experiments 1–3, the ^{65}Zn was injected into the tail vein, and the rats were killed 3 hours later. In experiment 4, the ^{65}Zn was mixed with the appropriate diets and fed in this form for 10 days before killing the rats. Four rats (exps. 2 and 3) or 5 rats (exps. 1 and 4) were used per treatment group.

Immediately after each rat was killed (by overanesthesia or decapitation), the liver was excised, blotted, and homogenized in 6 volumes (exp. 1) or 9 volumes (exps. 2–4) of ice-cold 0.05 M Tris · HCl

TABLE 2

Effects of zinc deficiency on the susceptibility of liver ^{65}Zn to dialysis

		Exp. 1	Exp. 2	Exp. 3	Exp. 4
Experimental diets fed, days		34	145	33	24
Wt of rats, g					
−Zn [1]		113 ± 5 [2]	223 ± 22 [2]	109 ± 1 [2]	86 ± 3 [2]
+Zn [1]		224 ± 7	409 ± 6	232 ± 1	167 ± 6
^{65}Zn administration		iv [3]	iv [3]	iv [3]	oral [4]
Dialyzing agent [5]	Dietary zinc [1]	Total ^{65}Zn removed from liver homogenates by dialysis against 8 successive 100-ml aliquots of external dialysis solution [6]			
EDTA	−Zn	64.3 ± 1.7 [d]	60.3 ± 1.2 [b]	71.9 ± 0.6 [b]	48.1 ± 1.0 [a]
	+Zn	76.8 ± 1.2	67.8 ± 2.9	78.9 ± 3.0	45.0 ± 1.7
CDTA	−Zn	53.5 ± 1.8 [d]	43.4 ± 1.0 [d]	62.1 ± 0.4 [c]	43.8 ± 0.8 [a]
	+Zn	67.8 ± 2.1	60.8 ± 3.0	72.6 ± 3.0	44.3 ± 1.7
Histidine	−Zn	39.1 ± 2.9 [b]	48.1 ± 2.7 [d]	48.3 ± 4.2 [a]	28.7 ± 3.1 [a]
	+Zn	26.1 ± 5.4	24.3 ± 3.4	50.3 ± 8.7	30.1 ± 0.8
Cysteine	−Zn	14.9 ± 1.4 [b]	13.0 ± 1.6 [b]	—	—
	+Zn	10.8 ± 1.2	8.1 ± 1.4	—	—
Glycine	−Zn	4.1 ± 1.2 [a]	2.5 ± 0.7 [a]	10.3 ± 1.7 [a]	2.0 ± 0.4 [a]
	+Zn	5.2 ± 1.2	4.0 ± 0.5	10.3 ± 1.8	3.2 ± 0.8
Buffer alone	−Zn	4.2 ± 1.5 [a]	1.8 ± 0.4 [a]	5.6 ± 2.9 [a]	1.8 ± 1.7 [a]
	+Zn	3.9 ± 0.3	2.3 ± 0.4	10.6 ± 2.3	3.0 ± 0.7
$ZnCl_2$-glycine	−Zn	—	—	90.7 ± 0.5 [a]	—
	+Zn	—	—	91.1 ± 0.8	—

[1] −Zn = rats fed the basal diet containing 1 ppm zinc; +Zn = rats fed the same diet supplemented with 20 ppm zinc.
[2] Treatment means ± SE. Four or five rats/treatment group.
[3] iv = ^{65}Zn injected into tail vein 3 hours before rats were killed.
[4] Oral = ^{65}Zn fed with diet for 10 days before rats were killed.
[5] Dialyzing agents used at a concentration of 0.001 M in 0.05 M Tris·HCl buffer, pH 7.4. EDTA = ethylenediaminetetraacetic acid; CDTA = *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid.
[6] Aliquots of liver homogenates were dialyzed for 192 hours at 5° or 15° (exp. 3) against successive 100-ml volumes of dialysis solution changed at 24-hour intervals. Values presented are treatment means ± SE for the total cumulative amount of ^{65}Zn removed. Superscript letters indicate significant differences between −Zn and +Zn values according to the following code:
a: $P > 0.10$; b: $0.05 < P < 0.10$; c: $0.01 < P < 0.05$; d: $0.001 < P < 0.01$.

buffer, pH 7.4. Aliquots of the homogenates were then pipetted into sacs prepared from 8-mm Visking dialysis tubing.[3] Although aliquot size varied from 3 to 10 ml between experiments because of differences in the amount of homogenate available and the number of dialyzing agents used, the volume of homogenate exposed to each dialyzing agent was uniform within a given experiment. The sacs were assayed for ^{65}Zn by use of a whole-animal gamma scintillation detector[4] and were then dialyzed in a cold room at 5° (exps. 1, 2 and 4) or at 15° (exp. 3) against 8 successive 100-ml aliquots of 0.001 M dialyzing agent in 0.05 M Tris · HCl buffer, pH 7.4. The following dialyzing agents were used: EDTA (ethlenediaminetetraacetic acid), CDTA (*trans*-1,2-diaminocyclohexane-N, N, N′, N′-tetraacetic acid), histidine, cysteine, glycine, and buffer alone. The synthetic chelating agents EDTA and CDTA were selected because of their high stability constants for the zinc-chelate complex (EDTA, 16.5; CDTA, 18.7 (11)). Histidine and cysteine were tested because of the presumed importance of imidazole and sulfhydryl groups in the physiological binding of zinc to proteins, and glycine was chosen as an agent with a low zinc affinity. In experiment 3, $ZnCl_2$ was also used as a dialyzing agent to measure the extent of ^{65}Zn removal by exchange.

The external dialysis solution was changed at 24-hour intervals, and the ^{65}Zn remaining in the sample after each successive 24-hour period was determined with the whole-animal gamma scintillation detector. A CDC 1604 computer was programed to accept the observed sample count rates after each dialysis period, apply corrections for coincidence loss, background, and radioactive decay, and express corrected count rates as a percentage of the ^{65}Zn initially present in the samples. The cumulative percentage removal of ^{65}Zn by a given dialyzing agent was then plotted as a function of time, and the data were analyzed for statistically significant effects of dietary zinc, using Student's *t* test (12).

RESULTS

As shown in table 2 and figures 1–4, the dialyzing agents used differed in effectiveness of ^{65}Zn removal from liver

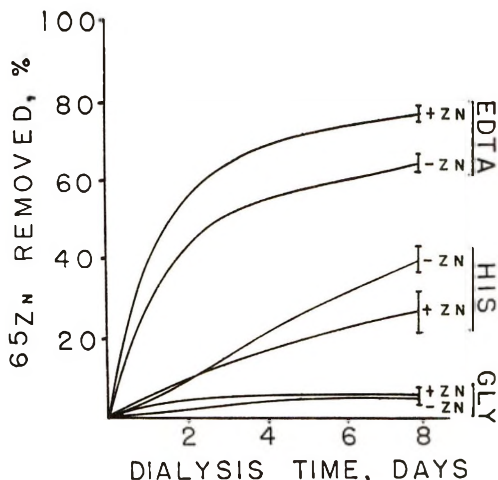

Fig. 1 Experiment 1: ^{65}Zn injected 3 hours before rats were killed. Effects of zinc deficiency on the susceptibility of liver ^{65}Zn to dialysis against 10^{-3} M ethylenediaminetetraacetic acid (EDTA), histidine (HIS), and glycine (GLY). − Zn = rats fed the basal low zinc diet (1 ppm Zn); + Zn = rats fed the same diet supplemented with 20 ppm zinc. Vertical bars for day 8 represent SE.

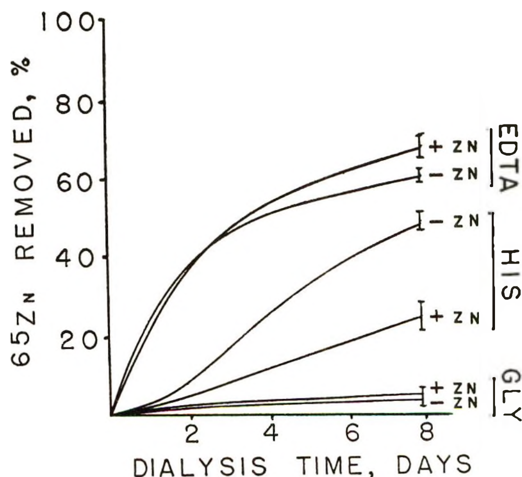

Fig. 2 Experiment 2: ^{65}Zn injected 3 hours before rats were killed. See legend for figure 1.

[3] Union Carbide Corporation, Chicago.
[4] Armac Scintillation Detector Model 440, manufactured by Packard Instrument Company, Inc., La Grange, Illinois.

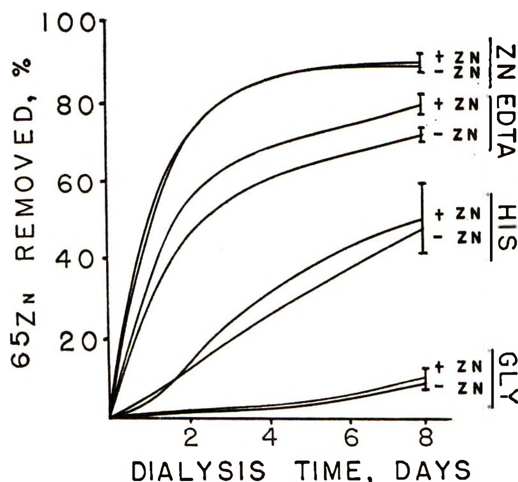

Fig. 3 Experiment 3: ^{65}Zn injected 3 hours before rats were killed. See legend for figure 1.

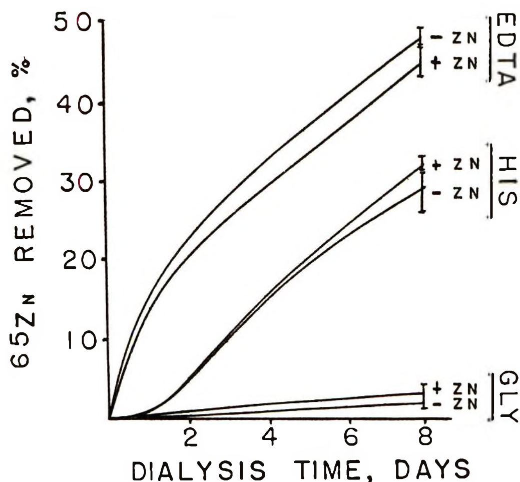

Fig. 4 Experiment 4: ^{65}Zn fed in diet for 10 days before rats were killed. See legend for figure 1.

homogenates in the order EDTA > CDTA > histidine > cysteine > glycine > buffer alone. The relative effectiveness of EDTA and glycine compares favorably with results reported by Dennes et al. (13) for dialysis of ^{65}Zn from plasma of ^{65}Zn-injected rabbits. The following compounds were also tested as dialyzing agents at a concentration of 10^{-3} M, but were found to be no more effective than glycine: anthranilic acid, lysine, glutamic acid, adenylic acid, and malonic acid. Little correlation was observed between the zinc affinity constants reported in the literature for these compounds and their effectiveness as dialyzing agents under the conditions used. Vohra and Kratzer (14) also concluded that stability constants reported in the literature are not always meaningful when applied to biological systems where conditions of pH and cation environment may differ from the conditions under which the values were originally determined.

In table 2 are presented the total amounts of ^{65}Zn removed by each dialyzing agent from liver homogenates prepared from rats fed either the −Zn or the +Zn diet. The removal of ^{65}Zn as a function of time is shown for each of the 4 experiments in figures 1–4. For clarity, the data for CDTA, cysteine, and buffer alone have been omitted from the figures, since these dialyzing agents had effects qualitatively similar to those with EDTA, histidine, and glycine, respectively. Although the results are discussed below for EDTA, histidine and glycine, similar statements could be made in all cases for CDTA, cysteine, and buffer alone.

In experiments 1–3 in which the ^{65}Zn was injected 3 hours before the rats were killed, EDTA (and CDTA) consistently removed a smaller percentage of ^{65}Zn from liver homogenates prepared from rats fed the −Zn rather than the +Zn diet (table 2). For EDTA, this effect was highly significant ($P < 0.005$) in experiment 1 and approached significance ($P < 0.10$) in experiments 2 and 3. In experiments 1 and 2, but not experiment 3, histidine (or cysteine) had the opposite effect: more injected ^{65}Zn was removed from the liver homogenates of −Zn rats than from those of +Zn rats. The higher temperature of the cold room in experiment 3 (15° vs. 5°) or possibly the difference in dietary amino acid source (that is, casein hydrolyzate vs. spray dried egg white) may account for the higher amounts of ^{65}Zn removed and the failure to observe an effect of zinc status of the rat on dialysis of liver ^{65}Zn against histidine.

Further differences between the synthetic chelating agents (EDTA and CDTA) and the amino acids histidine and cysteine are shown in kinetics of ^{65}Zn removal depicted in figures 1–3. The effects of

dietary zinc on the results obtained with glycine (or buffer alone) were uniformly nonsignificant.

The results are shown schematically in figure 5A, and they were obtained by averaging the results of experiments 1–3. Based on the susceptibility of injected ^{65}Zn to dialysis under the conditions of these studies and on the assumption that ^{65}Zn removal is dependent on the degree of zinc-binding by the dialyzing medium, total liver ^{65}Zn can be delineated into 4 pools. Pool A consists of ^{65}Zn which can be removed during 8 days of dialysis against glycine, pool B represents ^{65}Zn accessible to histidine but not to glycine, pool C contains ^{65}Zn which required EDTA to effect its removal but cannot be removed by histidine, and pool D is defined as that ^{65}Zn which cannot be removed by any of the dialyzing agents used, under the conditions employed in these studies. As indicated in figure 5A, the main effect of dietary zinc deficiency on the binding of injected ^{65}Zn by the liver at 3 hours after ^{65}Zn administration was on pool C, which accounted for a much smaller percentage of total liver ^{65}Zn in the liver homogenates prepared from rats fed the −Zn diet than in those prepared from rats fed the +Zn diet. This decreased amount of ^{65}Zn in pool C of the −Zn rats was accompanied by approximately equal increases in the amount of ^{65}Zn in both pools B and D. Pool C was smaller in the −Zn rats in each of the 3 experiments, although the effects were more marked in experiments 1 and 2 than in experiment 3.

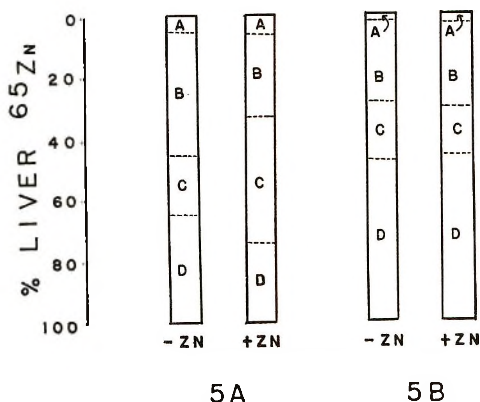

Fig. 5 Effects of dietary zinc deficiency on incorporation of ^{65}Zn into liver zinc pools. ^{65}Zn was either injected 3 hours before rats were killed (fig. 5A, average of exps. 1–3) or fed in the diet for 10 days before they were killed (fig. 5B, exp. 4). Based on the total amounts of ^{65}Zn removed during 8 days of dialysis of liver homogenates against 10^{-3} M EDTA, histidine, and glycine, liver ^{65}Zn was delineated into 4 pools, as follows: pool A, ^{65}Zn removed by glycine; pool B, ^{65}Zn removed by histidine, but not by glycine; pool C, ^{65}Zn removed by EDTA, but not by histidine and pool D, ^{65}Zn not removed by EDTA.

At least 2 explanations could be offered for the effects noted in experiments 1–3 and shown in figure 5A: Either the distribution of total liver zinc among binding sites of varying zinc affinity was altered in zinc deficiency, or the extent of incorporation of intravenously administered ^{65}Zn into the various pools during the 3 hours following injection was affected by the deficiency status of the rats. In the first case, figure 5A would represent actual difference in pool sizes due to zinc deficiency, and in the second case, pools would be unaltered in size, but would differ in the rate of labeling with injected ^{65}Zn.

To distinguish between these 2 possibilities, a fourth experiment was conducted, in which an attempt was made to label all liver zinc pools more or less uniformly with ^{65}Zn by allowing rats to consume a ^{65}Zn-containing diet for 10 days before they were killed. Selection of the 10-day labeling period was based on the rapid turnover of liver ^{65}Zn reported by other workers (15, 16). The results are shown in table 2 and in figures 4 and 5B, and can be readily summarized: none of the differences due to dietary zinc level was statistically significant for any of the dialyzing agents used at any of the time-periods during the 8 days of dialysis. These findings suggest that the effects of dietary zinc in experiments 1–3 resulted from differences in the rates of incorporation of ^{65}Zn into the various pools of liver zinc rather than from actual differences in total pool size.

DISCUSSION

The ability of various dialyzing agents to remove differing amounts of liver ^{65}Zn under the dialysis conditions used in these experiments is in accord with the hetero-

geneity of physiological binding sites which have been suggested for zinc. These include functional groups of amino acid residues in proteins and peptides, notably imidazole and sulfhydryl groups (17) and specific metal-binding sites of zinc metalloproteins (18) and of nucleic acids (19, 20).

Most of the ^{65}Zn present in the liver could not be removed by dialysis against 10^{-3} M glycine. In other studies,[5] it was shown that a tenfold increase in glycine concentration had little effect on the extent of ^{65}Zn dialysis from liver homogenate (< 12% removed) and that less than 12% of the ^{65}Zn could be recovered by dialysis against glycine when the ^{65}Zn was simply added to a liver homogenate in vitro rather than being injected into, or fed to, the rat before it was killed. This level of zinc binding, designated as pool A in figure 5, is apparently attributable to zinc which is present in, or readily equilibrated with, a very loosely bound form.

At the other end of the zinc-binding spectrum, more than 50% of the ^{65}Zn present in the liver homogenates prepared from rats fed ^{65}Zn for 10 days before they were killed could not be removed by dialysis against EDTA under the conditions described. Bartholomew et al. (21) have also reported that a fraction of liver ^{65}Zn could not be removed by EDTA. In all our experiments some liver ^{65}Zn was bound so firmly that it could not be removed by any of the dialyzing agents used; even after exhaustive dialysis against ionic zinc (exp. 4) or against 10^{-2} M EDTA,[6] about 10% of the initial ^{65}Zn remained in the homogenate aliquots. The possibility that this nondialyzable ^{65}Zn may have been bound to the dialysis tubing was excluded by flushing out the tubing at the end of the experiment and assaying it for radioactivity; routinely, less than 1% of the residual sample ^{65}Zn activity was found associated with the empty sac. Possible zinc-binding sites which might account for this pool of firmly bound zinc (pool D in fig. 5) include certain metalloproteins and the metal-binding sites of nucleic acids. For example, it is unlikely that the zinc in alcohol dehydrogenase can be removed with EDTA, since it is very firmly bound and treatment with reagents such as 1, 10-phenanthroline or 8-hydroxyquinoline-5-sulfonic acid is normally required to effect its removal, whereupon the enzyme either dissociates into subunits (22) or undergoes denaturation and aggregation.[7] Similarly, Wacker and Vallee (19) and Wacker et al. (20) have reported that some of the trace metals bound to RNA from various sources could not be removed by exposure to EDTA. Binding of zinc to the sites included in pool D apparently occurs only as a result of normal cellular metabolism, since ^{65}Zn mixed with a liver homogenate in vitro is almost completely removed by dialysis against Zn^{++}, EDTA, or CDTA, and the process is essentially complete after 4 to 6 days of dialysis under the conditions described above.[8]

The insensitivity of zinc concentration of rat liver to dietary zinc deficiency has been repeatedly reported in the literature (7–9) and confirmed in our laboratory.[9] Although zinc assays were not conducted on the livers used in the experiments reported here, we have, in numerous other studies,[10] analyzed livers from similar rats fed the diets shown in table 1. For rats comparable in size and deficiency status to those used in the present experiments, typical values for liver zinc concentration on a fresh-weight basis are (treatment means as μg/g ± SE) 32.1 ± 1.4 (−Zn) vs. 34.1 ± 2.3 (+Zn) and 31.7 ± 0.9 (−Zn) vs. 32.7 ± 1.0 (+Zn). In such comparisons, the difference in liver zinc concentration has always been small and has seldom been statistically significant. From the results of experiment 4 in the present report, it appears that the distribution of zinc among various cellular zinc-binding sites is similarly unaffected by the dietary zinc status of the rat. The apparent effect of zinc deficiency on the sizes of pools B, C and D (fig. 5) when the ^{65}Zn was injected 3 hours before the animals were killed is therefore probably the result of altered rates of incorporation

[5] Unpublished observations, W. M. Becker and W. G. Hoekstra.
[6] See footnote 5.
[7] Druyan, R., and B. L. Vallee 1962 Exchangeability of the zinc atoms in liver alcohol dehydrogenase. Federation Proc., *21:* 247 (abstract).
[8] See footnote 5.
[9] See footnote 5.
[10] See footnote 5.

of injected ^{65}Zn into liver zinc pools. Since the apparent decrease in pool C due to dietary zinc deficiency was accompanied by increases in both pools B and D, it is likely that at least 2 effects are involved.

A possible explanation for the increased amount of ^{65}Zn present in pool B in the zinc-deficient rats is that the greatly reduced growth rate of these rats at the time of ^{65}Zn injection resulted in a slower rate of labeling of sites in pool C which incorporate ^{65}Zn primarily because of *de novo* synthesis of binding sites rather than by means of rapid ^{65}Zn-Zn exchange reactions. The greater growth of the +Zn rats may, in other words, have resulted in more rapid ^{65}Zn incorporation into sites not accessible to histidine.

The incorporation of more ^{65}Zn into pool D by zinc-deficient rats than by zinc-supplemented rats during the 3 hours following intravenous ^{65}Zn injection suggests that the turnover rate of this pool is increased in zinc deficiency. Increased turnover of at least some proteins in zinc-deficient rats has recently been postulated by Hsu et al.[11] and an increased rate of RNA degradation in zinc-deficient organisms has also been suggested (8, 23). The assumption that incorporation of ^{65}Zn into this pool is dependent upon synthesis of the molecules responsible for this level of zinc binding is consistent with the observation that little or no ^{65}Zn was present in a form inaccessible to EDTA when the ^{65}Zn was simply mixed with the homogenate in vitro.

In conclusion, it appears that the binding pattern of liver zinc, like the total liver zinc concentration, is not altered in zinc deficiency in the rat, and therefore probably cannot serve as a means by which liver tissue can detect the overall deficiency status of the rat while still maintaining normal levels of tissue zinc. In particular, the pool of loosely bound zinc (pool A), which might have been expected to respond most readily to the deficiency, was not significantly depleted in the −Zn rats, according to the results obtained by dialysis of liver ^{65}Zn against glycine or buffer alone. However, the rate of incorporation of zinc into firmly bound forms was affected by the zinc deficiency, resulting in an apparent decrease in the amount of liver ^{65}Zn which could be removed by EDTA, but not by histidine, under the dialysis conditions used in these studies.

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Time-Course of Changes in Rat Liver Enzyme Activities after Initiation of a High Protein Regimen [1]

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ABSTRACT The effects of feeding a high protein diet to rats previously adapted to a high carbohydrate diet containing adequate protein was studied in male Sprague-Dawley rats. The high carbohydrate diet was found to stimulate growth which was not augmented noticeably by feeding the high protein diet. Liver protein reached maximal values one day after the dietary change, whereas relative liver size values remained relatively constant. The glycogen content of liver was minimum one day after the dietary change which was followed by relatively higher and constant values. The enzymes studied could be divided into 2 groups based on the pattern of change in enzyme activity after the dietary shift. In one group, which includes fructose 1,6-diphosphatase, glucose 6-phosphatase, glutamic-oxaloacetic transaminase, glutamic-pyruvate transaminase, serine dehydrase and tyrosine-α-ketoglutaric transaminase, enzyme activity increased with time. Furthermore, the activity of these enzymes increased without a lag, with the exception of fructose 1,6-diphosphatase, which increased in activity only after a one-day delay. In the second group, which includes L-α-glycerophosphate dehydrogenase, glucose 6-phosphate dehydrogenase, pyruvate kinase and malic enzyme, enzyme activity was decreased by feeding the high protein diet, except that the activities of pyruvate kinase and malic enzyme were temporarily increased one day after the dietary change. The physiological significance of a lag in enzyme induction, when such occurs, and a possible mechanism for the transitory induction of pyruvate kinase and malic enzyme by the high protein diet are discussed.

In a previous publication (1), the time-course of induction of several rat liver enzymes after a non-protein to high protein dietary shift was described. The treatment was found to increase the activities of most of the enzymes studied, even the activities of those enzymes involved in the metabolism of carbohydrates. Two patterns of induction were distinguishable; in one pattern enzyme activity reached a maximum within 48 hours after the dietary change and in the other pattern induction was preceded by a 24-hour lag and the time-course of induction appeared to be sigmoidal over a period of 7 days.

It was suspected that the lag in induction was due to some derangement in the synthetic apparatus of the liver. Furthermore, it appeared probable that the transitory elevation in the activity of pyruvate kinase and malic enzyme after the feeding of the high protein diet was caused primarily by a sudden spurt in the synthesis of these enzymes due to the influx of amino acids into the liver. Such influx of amino acids could conceivably increase protein synthesis by stimulating reaggregation of polysomes on messenger RNA already present (2). If these assumptions were correct, then a dietary shift from a high carbohydrate diet which contains adequate protein to a high protein diet which contains no carbohydrate should increase the synthesis of amino acid-metabolizing enzymes without a lag. The transitory increase in pyruvate kinase and malic enzyme activities, however, may still occur as a consequence of the increase in dietary protein and hence, amino acid concentration in the liver. To test the validity of these assumptions the following experiments were undertaken.

EXPERIMENTAL

Male rats of the Spague-Dawley strain were fed a high carbohydrate diet consisting of: (in percent) casein, 25; glucose, 65; corn oil, 5; minerals (3), 4; and vitamins (4), 1. Four days later the animals were offered a diet in which casein was substituted for glucose, but which was otherwise identical to the previous diet.

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The animals were killed in the early morning at zero, 1, 2, 3, 4 and 7 days following the change to the high protein, carbohydrate-free diet. The animals had been housed individually in screen-bottom cages and offered food and water ad libitum.

The preparation of liver homogenates, the determination of liver protein and glycogen and the detemination of enzyme activities were previously described (1, 5).

RESULTS

The effects of the high protein diet on body weight and liver constituents are summarized in table 1. The animals gained a considerable amount of weight after being fed the 65% glucose, 25% casein diet for 4 days. This gain in body weight was not augmented by feeding the high protein diet. Both of these observations are consistent with earlier reports (6) that near maximal growth can be achieved in rats fed 20 to 40% protein in the diet and that further increase in the concentration of protein will not increase, or may even decrease, the growth rate. It was also observed that the animals exhibited a tendency to reject the high protein diet 4 days after the dietary change, which perhaps accounts for the decrease in liver parameters by the seventh day of feeding the high protein regimen.

Liver protein values, both total and soluble, had reached maximal values one day after the feeding of the high protein diet began. In contrast with this, relative liver size values did not change significantly until after 4 days, when a decline in these values was observed. As expected from previous work (1), there was a sharp drop in liver glycogen content on the first day after the dietary change. This was followed by higher and relatively constant values until the seventh day, when liver glycogen was almost nonmeasurable.

These results indicate that the physiological consequences of this dietary shift (from adequate to high protein) are much less severe than those encountered in the shift from protein-free to high protein diet (1).

It has been reported previously that the activities of serine dehydrase (7, 8), glutamic-pyruvic transaminase (9–11), glutamic-oxaloacetic transaminase (9, 10, 12, 13) and tyrosine-α-ketoglutarate transaminase (14) are increased by dietary protein. In our experiments the activities of these enzymes were increased by the dietary shift (table 2) and the lag observed in the protein-free to high protein shift was not observed. This is in agreement with the assumption that the synthetic apparatus of the liver is deranged by feeding a protein-free diet and that the lag observed in the induction of these enzymes when protein is refed may be due to the repair of the synthetic apparatus (1). It was expected that the activities of glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase and serine dehydrase would be much higher in the animals fed the high carbohydrate, adequate-protein diet as compared with the animals fed the high carbohydrate, protein-free diet (1). Althouth this expectation was partially realized, the differences in enzyme activities were not as great as expected. Thus it appears that the effect on the synthetic apparatus of liver (that is, the lag in induction of the transaminase and serine dehydrase upon refeeding protein to the animals fed the protein-free diet) caused by feeding the high carbohydrate, protein-free diet, is not due to the presence of glucose, but to the absence of protein in the diet.

The activity of fructose 1,6-diphosphatase, which is increased by dietary protein (1, 15), was induced in these experiments only after the second day of feeding the high protein diet. The observed lag in induction indicates that the control of synthesis of this enzyme must involve some factors (which cause the lag) other than those involved in the control of the transaminases and serine dehydrase.

The effects of the dietary shift on a number of carbohydrate-metabolizing enzymes and malic enzyme are summarized in table 2. To compare the changes in these enzymes on a percentage basis the activities after 4 days of feeding the high carbohydrate diet were defined as 100%. Figure 1 shows that L-α-glycerophosphate dehydrogenase and glucose 6-phosphate dehydrogenase activities were decreased by increasing the protein content of the diet. Malic enzyme and pyruvate kinase activities were also decreased, but the decrease was preceded by a transitory

TABLE 1

Effect of high protein diet on several liver constituents and body weight

No. of days high protein diet fed	0	1	2	3	4	7
No. of animals/group	6	6	8	8	8	6
Original body wt before feeding 65% glucose diet, g	159 ± 2 [1]	172 ± 7	165 ± 5	169 ± 4	175 ± 5	167 ± 14
Body wt at killing, g	184 ± 4	197 ± 6	199 ± 5	200 ± 6	203 ± 7	208 ± 5
Total liver protein, mg/100 g body wt	755 ± 14	1040 ± 71	1050 ± 100	1090 ± 56	853 ± 70	827 ± 79
Soluble liver protein, mg/100 g body wt	504 ± 30	672 ± 40	658 ± 10	653 ± 25	580 ± 30	491 ± 20
Liver glycogen, mg/100 g body wt	152 ± 16	26.4 ± 13	99.4 ± 11	94.9 ± 19	98.0 ± 20	< 10
Relative liver size = (wt of liver) × 100/(body wt at killing)	4.80 ± 0.5	4.98 ± 0.2	4.85 ± 0.1	4.92 ± 0.2	4.67 ± 0.3	3.81 ± 0.3

[1] SE of mean.

TABLE 2

Effect of high protein diet on rat liver enzymes [1]

No. of days high protein diet fed	0	1	2	3	4	7
No. of animals/group	6	6	8	8	8	6
Fructose 1,6-diphosphatase	13.6 ± 0.57 [2]	10.3 ± 2.8	19.7 ± 2.4	24.2 ± 1.5	18.0 ± 1.3	12.4 ± 1.6
Glucose 6-phosphatase	60.0 ± 7.0	110 ± 9.3	81.4 ± 4.8	88.1 ± 13	75.3 ± 4.1	69.3 ± 1.5
L-α-glycerophosphate dehydrogenase	242 ± 19	255 ± 11	207 ± 8.4	194 ± 15	189 ± 15	140 ± 12
Glucose 6-phosphate dehydrogenase	65.1 ± 6.4	61.5 ± 5.9	32.8 ± 3.4	31.8 ± 3.4	23.6 ± 5.0	12.4 ± 1.4
Pyruvate kinase	120 ± 17	178 ± 16	107 ± 7.0	113 ± 14	71.1 ± 9.8	51.8 ± 6.4
Malic enzyme	11.7 ± 2.3	28.2 ± 6.3	9.59 ± 1.6	7.64 ± 1.5	6.04 ± 1.3	1.44 ± 0.17
Glutamic-oxalacetic transaminase	236 ± 13	286 ± 26	267 ± 16	398 ± 23	361 ± 39	272 ± 31
Glutamic-pyruvic transaminase	33.0 ± 4.5	54.5 ± 7.9	80.9 ± 8.9	98.8 ± 3.5	112 ± 7.6	101 ± 15
Serine dehydrase	3.06 ± 0.33	9.21 ± 0.94	17.1 ± 3.7	19.6 ± 1.8	23.6 ± 1.9	13.7 ± 1.7
Tyrosine-α-ketoglutaric transaminase	1.20 ± 0.13	2.98 ± 0.24	5.44 ± 0.70	5.67 ± 1.0	4.68 ± 0.75	1.17 ± 0.61

[1] Values of enzyme activities are given as μmoles of substrate converted/minute/100 g body weight.
[2] SE of mean.

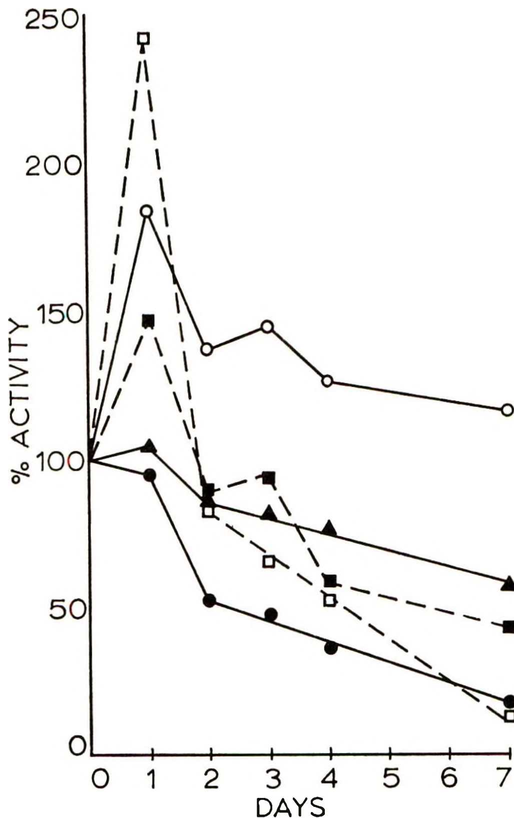

Fig. 1 Time-course of increase of rat liver enzymes. The diet was changed from one containing 65% glucose and 25% casein to a diet consisting of 90% casein and free of carbohydrate. Enzyme activity after 4 days of feeding the high carbohydrate diet was defined as 100%. Glucose 6-phosphatase, ○———○; glucose 6-phosphate dehydrogenase, ●———●; L-α-glycerophosphate dehydrogenase, ▲———▲; malic enzyme (TPN)□———□; and pyruvate kinase ■———■.

increase on the first day of feeding the high protein regimen. In comparison, glucose 6-phosphatase activity reached a maximum one day after the dietary shift and began to decline afterwards, though the activity of this enzyme was still higher than before the dietary shift.

The induction of glucose 6-phosphate dehydrogenase has been shown to be dependent on dietary protein (16–20) and carbohydrate (21). Possibly, however, the control of this enzyme is very sensitive to a number of factors and, therefore, is very complicated. The reported inability of dietary protein (free of carbohydrate) to induce the activity of this enzyme after starvation (22) contrasts sharply with results reported in an earlier communication (1), where a carbohydrate-free, high protein diet did induce glucose 6-phosphate dehydrogenase after pre-feeding with a protein-free diet. We have also been observing a slight induction of this enzyme with a high protein, carbohydrate-free diet in meal-fed rats.[2] However, in every case in which dietary protein induced the activity of this dehydrogenase the pre-induction level of the enzyme was very low, whereas in the present experiments the activity of glucose 6-phosphate dehydrogenase was rather high before the feeding of the high protein diet. And although there can be no doubt that a primary inducer of this enzyme is glucose, glucose 6-phosphate dehydrogenase may also be induced by protein, starting from very low levels of enzyme activity and after negative nitrogen balance, but not after starvation.

It is of interest that the activities of glucose 6-phosphate dehydrogenase and L-α-glycerophosphate dehydrogenase respond similarly to the dietary change. This might be expected, as it has been suggested that these enzymes may be closely linked to lipogenesis (5). However, the control of the activities of these enzymes is not always the same as shown in a previous paper (1).

We have previously reported a transitory elevation in the activities of pyruvate kinase and malic enzyme shortly after the feeding of a high protein diet to animals pre-fed with a protein-free diet. The same tendency was observed when the animals were pre-fed with a high carbohydrate diet containing adequate protein (fig. 1). This increase cannot be attributed to increased internal concentrations of glucose, as such would induce glucose 6-phosphate dehydrogenase as well. Increased activities of pyruvate kinase and malic enzyme, therefore, are probably brought about by increasing the rate of synthesis at the translational level. It is of interest that the transitory elevation in the activity of these 2 enzymes disappears within a day, suggesting that the half-life of these enzymes is less than 24 hours. A value of the half-life of malic enzyme, reported to be 4

[2] Szepesi, B., and R. A. Freedland, unpublished data.

days (23), then appears too high; from our data and the data obtained by Shrago and co-workers (24), the half-life of malic enzyme appears to be less than 24 hours.

DISCUSSION

The observed lag in the induction of the transaminases after re-feeding protein to animals pre-fed with a protein-free diet may have an important function in the maintenance of homeostasis and particularly in the conservation of nitrogen. It is known that withholding of dietary protein results in negative nitrogen balance and the conservation of nitrogen becomes of prime importance. If such animals are re-fed with a diet containing protein, such protein must first be used to replenish the nitrogen loss which occurred during the negative nitrogen balance. Such rebuilding of body protein would be hampered by an immediate and substantial increase in the activity of transaminases and serine dehydrase. However, rats prefed with a high carbohydrate diet which contains adequate protein do not develop a negative nitrogen balance; and an immediate and substantial increase in transaminase levels after the feeding of a high protein diet helps to maintain homeostasis by promoting nitrogen excretion and the conversion of amino acid skeletons into fats and carbohydrates.

The decrease in the activities of certain carbohydrate-metabolizing enzymes during gluconeogenic conditions (as feeding a high protein diet) also appears to have a physiological significance. That is, when it is of prime importance that the liver produce an increased amount of glucose, a decrease in the activity of some enzymes involved in glucose breakdown (particularly those which might reverse gluconeogenesis or promote the utilization of glucose in pathways other than gluconeogenesis) would be beneficial. Hence, glucose 6-phosphate dehydrogenase, pyruvate kinase and L-α-glycerophosphate dehydrogenase are "low priority enzymes" under such conditions. It could be argued that the transitory increase in malic enzyme activity may be important by converting pyruvate to malic acid which would provide an additional driving force for gluconeogenesis through increasing oxaloacetate concentration. The concurrent increase in pyruvate kinase activity, however, makes the defense of such a mechanism difficult. Based on available data however, our previous suggestion that the transitory increase in pyruvate kinase and malic enzyme activities (after increasing the protein content of diet) is mediated at the translation level (1) appears very probable. Such increases would simply be mediated by maximal loading of transfer RNA's as the amino acid content of blood and liver increases following the feeding of a high protein regimen before the messenger RNA's for these enzymes are degraded as protein feeding is continued. However, evidence available is not sufficient to ascertain this possibility.

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Recovery of Rat Tissue Lipids from Essential Fatty Acid Deficiency: Brain, heart and testes [1]

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ABSTRACT To study the ability of different tissues to recover from essential fatty acid deficiency, male rats were maintained for 25 weeks with a diet containing 10% hydrogenated coconut oil and were then transferred to a diet containing 10% corn oil. The total fatty acid compositions of brain, heart and testes lipids were determined at intervals over an 81-day period. Lipids from the deficient animals were low in the linoleic-series acids and high in oleic-series acids. Feeding corn oil to the deficient animals reversed this situation. Linoleic acid was rapidly incorporated into heart lipids, the changes in arachidonic and the higher polyunsaturated acids occurring more slowly. Slightly elevated levels of eicosatrienoic persisted after the concentration of total ω6-acids had returned to normal. Changes in brain fatty acids were slow, reflecting the slow metabolic turnover of lipids in this tissue. Docosahexaenoic acid, which is relatively high in brain lipids, was independent of the initial diet or of the supplemental corn oil diet. Arachidonic acid was more readily incorporated into the testes lipids than any other acid. Docosapentaenoic acid, the major ω6-acid of the testes from control animals, remained at a constant low level for several weeks before increasing to a level greater than that of the controls at 81 days. Both tissue and fatty acid specificities were observed.

Diets low in linoleic acid induce modifications in the fatty acid composition of most tissues and these changes are well-documented (1, 2). Comprehensive data on the sequential changes in the fatty acids from different tissues are found less frequently in the literature. In a previous publication (3) the changes in the total fatty acids from the liver, plasma and erythrocytes of essential fatty acid-deficient rats re-fed corn oil for 81 days were reported. The present communication extends this data to the brain, heart and testes of the same animals. These tissues were chosen for the diversity of polyunsaturated fatty acids present in them.

MATERIALS AND METHODS

The basic experimental procedures were those used previously (3). Male rats of the Wistar strain were maintained with diets containing 68% dextrose, 18% casein, salt and vitamin supplements, and 10% hydrogenated coconut oil.[2] Corn oil (10%) was substituted for coconut oil in the diet of the control group. After 25 weeks, the experimental group was transferred to the corn oil diet. Groups of 3 animals were killed at various intervals over an 81-day period and the tissues were immediately chilled.

The tissues were homogenized and extracted 3 times with 20 volumes of 2:1 (v/v) chloroform-methanol. The combined extracts were washed with 0.2 volumes of water to remove water-soluble components, and the aqueous phase was removed after centrifugation and the extract was dried over sodium sulfate before removal of the solvent *in vacuo*.

Esterification was effected by refluxing 10 to 20 mg of the lipids with 1.0 ml of benzene and 4.0 ml of 15% borontrifluoride in anhydrous methanol, the esters were purified by thin-layer chromatography and analyzed by gas-liquid chromatography. A Barber-Colman series 5000 gas chromatograph equipped with dual columns and dual hydrogen flame ionization detectors was used. The stainless steel columns, 150 cm × 2 mm I.D. were packed with 15% ethyleneglycol succinate-silicone copolymer (EGSS-X) on 80–100 mesh Chromosorb W and were programed from

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160 to 200° at 4° per minute. Peak area was measured as the product of peak height and width at half-peak height and the detector was calibrated against a quantitative standard [3] containing saturated esters of even chain length from C_{14} to C_{24}. Experimental and theoretical concentrations agreed within a mean relative error of 2%.

RESULTS

The dieary corn oil was rich in linoleic (58.8%) and oleic (26.5%) acids, with stearic and palmitic constituting 2.0% and 11.2%, respectively, of the total fatty acids. The hydrogenated coconut oil contained less than 0.1% linoleic and only 0.2% oleic acid. Lauric (49.4%), myristic (18.4%), palmitic (9.8%) and stearic (11.7%) were the major acids in this fat.

The major unsaturated fatty acid components of the total brain lipids from rats fed the control diets and during the period of supplemental feeding of the deficient animals with corn oil are presented in table 1. Brain lipids of the animals fed the corn oil diet were characterized by relatively high levels of the ω9-acids (25.5%) and lower levels of the ω6-acids (18.3%). Arachidonic acid was the major ω6-acid present with lesser amounts of the 22:4 and 22:5 acids of the series. Linoleic acid was a relatively minor component of the brain lipids of this group.

Unlike the other tissues examined, high levels of docosahexaenoic acid (22:6ω3) [4] were found. This acid, characteristic of the brain lipids of many species (4–6), accounted for approximately 10% of the total fatty acids, and its dietary precursor, linolenic acid, made up almost 4% of the total fatty acids in the brain. The saturated acids, palmitic and stearic, accounted for 20.1% and 21.8%, respectively, of the total fatty acids of brain lipids.

Feeding coconut oil in place of corn oil had very little effect on the ω3-acids of the brain. In fact, the brain lipids were less susceptible to dietary changes than any of the other tissues studied. This is undoubtedly due to the early stage in life at which these lipids are formed and to their relative metabolic stability. Decreases were observed in the ω6-acids, and these were accompanied by increased levels of the ω9-acids, primarily 20:3ω9, and 22:3ω9, when coconut oil was fed.

Once established, the fatty acid patterns resulting from the coconut oil diet changed very slowly when corn oil was substituted for the former dietary fat. The eicosatrienoic acid and 22:3ω9 decreased very slowly and even after 81 days of supplemental feeding were present at concentrations above those observed in the corn oil control animals. The accumulation of the ω6 polyunsaturated fatty acids, 20:4 and 22:4, was also a slow process, which again attests to the relative metabolic stability of the brain lipids.

Linoleic was the major unsaturated acid in the heart lipids from rats fed corn oil (table 2). Arachidonic accounted for 18.9% and the pentaenoic acid of the ω6-series, 22:5, was present to the extent of 4.5% of the total fatty acids. The deficient diet resulted in elevated levels of 20:3ω9 (25%), oleic and palmitoleic acids, whereas the levels of linoleic and arachidonic acids were considerably depressed. The levels of palmitic (13%) and stearic (18%) were independent of the diet.

The heart lipids from the deficient rats responded rapidly to the corn oil diet. Linoleic acid attained the level observed in the corn oil control group within 6 days and oleic acid decreased to the control level in 6 to 9 days. Changes in the other unsaturated acids occurred more slowly. Arachidonic increased to the level in the corn oil controls in 15 days and palmitoleic acid required 15 to 22 days to reach the control level. Although 20:3ω9 decreased rapidly in the initial 22 days of supplemental feeding, slightly elevated levels of this acid persisted for somewhat longer (0.2% at 63 days). Similarly, the concentrations of the highly unsaturated acids, 22:4ω6 and 22:5ω6, increased slowly on feeding corn oil.

In table 3 are presented the data for rat testes lipids. Testes from rats fed

[3] Methyl ester standard H-104, Applied Science Laboratories, Inc., State College, Pennsylvania.

[4] X:YωZ, notation for chain length, number of double bonds, and number of carbon atoms from center of ultimate double bond to, and including, the terminal methyl group in fatty acids.

TABLE 1

Effect of corn oil diet on the unsaturated fatty acids of brain total lipids from EFA-deficient rats [1]

Acid [2]	Corn oil control	Coconut oil control	Days fed corn oil diet				
			6	9	22	63	81 [3]
	% of total fatty acids		*% of total fatty acids*				
18:1ω9	25.3 ± 0.36	27.5 ± 1.06	25.8 ± 0.06	24.8 ± 0.01	24.7 ± 0.47	23.9 ± 0.61	23.9 ± 1.35
18:2ω6	1.6 ± 0.69	0.3 ± 0.10	0.9 ± 0.23	0.9 ± 0.01	1.8 ± 0.67	1.1 ± 0.17	2.2 ± 1.41
18:3ω3	3.7 ± 0.18	3.0 ± 0.10	3.2 ± 0.15	3.0 ± 0.01	2.6 ± 0.15	3.3 ± 0.32	3.1 ± 0.80
20:3ω9	0.2 ± 0.07	6.9 ± 0.35	5.2 ± 0.14	4.5 ± 0.03	2.9 ± 0.09	1.1 ± 0.00	0.8 ± 0.05
20:4ω6	10.4 ± 0.12	5.5 ± 0.19	7.4 ± 0.03	7.8 ± 0.21	10.0 ± 0.28	10.3 ± 0.12	11.4 ± 1.85
22:3ω9	tr [4]	1.6 ± 0.15	2.0 ± 0.17	2.1 ± 0.06	1.4 ± 0.21	0.6 ± 0.06	0.3 ± 0.05
22:4ω6	3.4 ± 0.15	1.1 ± 0.10	1.6 ± 0.09	1.9 ± 0.03	2.4 ± 0.18	4.1 ± 0.18	3.9 ± 0.45
22:5ω6	2.7 ± 0.26	2.2 ± 0.26	3.2 ± 0.24	2.7 ± 0.07	2.8 ± 0.13	2.8 ± 0.20	3.5 ± 0.25
22:6ω3	9.2 ± 0.31	9.6 ± 0.66	9.3 ± 0.24	9.4 ± 0.12	8.1 ± 0.43	9.0 ± 0.37	7.8 ± 0.20
Total ω6 [5]	18.3	9.1	13.7	13.9	17.5	18.6	21.3
Total ω9 [5]	25.5	36.0	33.0	31.4	29.0	25.0	25.0

[1] Mean of 3 animals ± SE except as noted. Minor components omitted from table.
[2] X:YωZ, notation for chain length, number of double bonds, and number of carbon atoms from center of ultimate double bond to, and including, the terminal methyl group in fatty acids.
[3] Mean of 2 animals.
[4] Trace, < 0.1%.
[5] Includes higher polyunsaturated acids.

TABLE 2

Effect of corn oil diet on the unsaturated fatty acids of heart total lipids from EFA-deficient rats [1]

Acid	Corn oil control	Coconut oil control	Days fed corn oil diet				
			2	6	9	15	22
	% of total fatty acids		*% of total fatty acids*				
16:1ω7	0.7 ± 0.15	4.7 ± 0.25	3.1 ± 0.18	2.2 ± 0.09	1.6 ± 0.06	1.0 ± 0.07	0.8 ± 0.03
18:1ω9	13.1 ± 0.45	28.2 ± 0.50	19.0 ± 0.58	14.7 ± 0.00	13.9 ± 0.56	11.4 ± 0.46	11.4 ± 0.43
18:2ω6	27.0 ± 0.92	2.8 ± 0.15	14.8 ± 0.38	27.2 ± 0.54	25.8 ± 1.13	28.5 ± 1.02	27.4 ± 0.76
20:3ω9	tr [2]	25.0 ± 0.91	16.4 ± 0.41	9.0 ± 0.32	5.9 ± 0.47	2.3 ± 0.20	1.0 ± 0.02
20:4ω6	18.9 ± 0.78	4.8 ± 0.28	9.6 ± 0.49	10.9 ± 0.15	16.2 ± 0.67	18.2 ± 1.00	19.4 ± 0.32
22:4ω6	1.6 ± 0.12	0.2 ± 0.03	0.3 ± 0.09	0.5 ± 0.03	0.6 ± 0.03	1.0 ± 0.06	1.4 ± 0.12
22:5ω6	4.5 ± 0.45	0.3 ± 0.06	0.6 ± 0.07	1.1 ± 0.20	1.5 ± 0.32	2.5 ± 0.27	3.4 ± 0.38
Total ω6 [3]	52.3	8.1	26.6	40.7	44.8	50.7	51.9
Total ω9 [3]	13.1	53.7	35.9	24.2	20.0	13.9	12.6

[1] Mean of 3 animals ± SE. Minor components omitted from table.
[2] Trace, < 0.1%.
[3] Includes higher polyunsaturated acids.

TABLE 3

Effect of corn oil diet on the unsaturated fatty acids of testes total lipids of EFA-deficient rats[1]

Acid	Corn oil control	Coconut oil control	Days fed corn oil diet					
			6	9	22	41	63	81[2]
	% of total fatty acids		*% of total fatty acids*					
16:1ω7	1.7 ± 0.23	5.8 ± 2.29	6.2 ± 0.74	4.4 ± 1.99	4.6 ± 0.71	4.5 ± 1.08	3.9 ± 1.21	1.8 ± 0.25
18:1ω9	14.9 ± 0.83	28.2 ± 0.80	25.0 ± 1.22	21.6 ± 4.53	22.6 ± 2.39	20.3 ± 1.64	20.1 ± 2.82	15.4 ± 0.15
18:2ω6	9.4 ± 2.13	0.6 ± 0.00	4.6 ± 1.05	4.3 ± 0.48	6.7 ± 0.55	8.2 ± 0.50	9.3 ± 2.02	7.0 ± 0.05
20:3ω9	0.1 ± 0.06	9.5 ± 0.40	3.1 ± 0.50	2.4 ± 0.81	0.8 ± 0.19	0.2 ± 0.06	0.2 ± 0.03	tr[3]
20:4ω6	14.4 ± 1.18	4.9 ± 0.78	11.2 ± 3.19	15.3 ± 2.04	15.4 ± 1.64	16.3 ± 1.01	14.9 ± 1.39	15.2 ± 0.35
22:3ω9	tr	2.4 ± 0.26	1.4 ± 0.34	1.1 ± 0.44	0.8 ± 0.19	tr	tr	tr
22:4ω6	2.0 ± 0.09	1.9 ± 0.64	1.4 ± 0.28	1.6 ± 0.06	2.4 ± 0.42	2.2 ± 0.13	1.3 ± 0.30	2.0 ± 0.55
22:5ω6	17.9 ± 1.75	7.9 ± 0.02	6.7 ± 1.37	7.7 ± 2.35	8.8 ± 1.61	10.0 ± 2.29	14.3 ± 3.90	21.7 ± 0.15
Total ω6[4]	44.6	15.3	24.7	29.5	34.1	37.4	40.4	46.0
Total ω9[4]	15.0	40.1	30.0	25.1	24.2	20.5	20.3	15.4

[1] Mean of 3 animals ± SE except as noted. Minor components omitted from table.
[2] Mean of 2 animals.
[3] Trace, < 0.1%.
[4] Includes higher polyunsaturated acids.

corn oil were characterized by high concentrations of 22:5ω6, which accounted for almost 18% of the total fatty acids. High levels of this acid have been reported in mature rat testes by several workers (7, 8). This was the major unsaturated acid in this tissue. Arachidonic and linoleic constituted 14 and 9%, respectively, of the total fatty acids. As was to be expected, dietary coconut oil depressed the levels of all the ω6-series acids and resulted in elevated levels of the ω9- and ω7-acids. The greatest increase was found in the concentration of oleic acid; eicosatrienoic acid accounted for less than 10% of the total acids of this group. A docosatrienoic acid of the ω9-series was present. This acid was also found in brain and erythrocyte lipids (3) from the animals deficient in essential fatty acids (EFA).

The effects of the corn oil diet on the testes fatty acids from the deficient animals differed from those observed with the other tissues investigated. The changes generally occurred more slowly than in the other tissues. Oleic and palmitoleic acids remained above the control levels until the rats had been fed corn oil for at least 63 days, and 20:3ω9 and 22:3ω9 did not approach control levels for at least 29 days. The increase in linoleic acid concentration was also slow, and 22:5ω6 concentration in the testes remained almost constant for 22 days before starting a slow increase followed by a more rapid increase after 63 days. In animals killed after 81 days, the concentration of this acid exceeded that observed in the control animals. In contrast with the slow changes noted above, arachidonic acid increased rapidly when corn oil was fed to these rats, reaching the control level in 9 days.

DISCUSSION

Incorporation of the ω6-acids into the brain was a slow process, extending over the 81 days of the experiment and reflecting the very low metabolic turnover of the brain lipids. There did not appear to be any preferential incorporation of linoleic acid over the polyunsaturates as observed with all of the tissues studied previously (3) and with the heart lipids. However, the low level of linoleic acid found in the

brain lipids of the control animals renders assessment of any preferential incorporation of this acid difficult in the present experiments. The brain also differed from all of the other tissues studied in that the oleic acid content changed only slightly when coconut oil was fed in place of corn oil. The major changes occurred in the polyunsaturated fatty acids. This has been noted by other workers (5, 9).

Rapid changes in linoleic and oleic acids in heart lipids of deficient animals refed linoleic acid were noted by Nervi and Brenner (10). In contrast with the results presented above, Nervi and Brenner reported relatively slow changes in the 20:4ω6 and 20:3ω9 acids during linoleic acid supplementation. However, they fed much lower levels of linoleic acid (110 mg/day) than used in the present experiment (0.7 to 1.0 g of linoleic acid/day). In experiments using arachidonic acid supplementation (10), changes in arachidonic acid levels in heart lipids were more rapid, but loss of eicosatrienoic acid was more gradual, again resembling the changes noted in the present study.

The results obtained above for changes in testicular fatty acid patterns are of interest in relation to the function of this tissue and to the effects of EFA-deficiency on this function. Male rats fed EFA-deficient diets become sterile (11) and the testicular tissue undergoes degeneration (12). Davis et al. (8) showed that the 22:5ω6 content of rat testicular phosphatides increased as the animals aged and that this increased in pentaenoic acid accompanied the appearance and maturation of the spermatids. Vitamin E deficiency in the rat, which also results in testicular degeneration and sterility, resulted in a marked decrease in the amount of 22:5ω6 in the testicular lipids (13). The lack of incorporation of this polyunsaturated acid in the early stages of supplemental corn oil feeding in the present study could have resulted from inhibition of certain enzyme systems due to the breakdown in structural components of the testes induced by EFA-deficiency. It is of interest that in the work reported by Bieri and Andrews (13) arachidonic acid accumulated in testes from vitamin E-deficient animals. In the present study, the level of this acid increased rapidly during the period of essential fatty acid supplementation.

Evans et al. (12), in their experiments on male sterility, observed marked degeneration of the testes after they fed rats a fat-free diet for 6 months, about the same period as in the present study. The sterility and degeneration of the testes was reversed by supplementing the diet of these animals with linoleic acid for periods from 9 to 15 weeks. No attempts were made to determine the fertility of the male rats in the present study; but the marked upturn in the concentration of 22:5ω6 at about the ninth week of supplemental feeding coupled with the previously demonstrated association of this acid with sexual maturity (8) is of interest in this respect.

The results obtained for the fatty acid patterns of the testes lipids during supplemental feeding with corn oil were variable, particularly in the case of the 22:5ω6 acid. For example the concentration of this acid varied from 3.2 to 11.1% in the 9-day sample, and from 7.6 to 21.1% in the animals killed after 63 days of supplemental feeding. Similar variations in oleic and arachidonic acids occurred in some instances. This was taken as an indication of either differences in the susceptibility of individual animals to essential fatty acid deficiency or of differences in their ability to recover from the deficiency. It is noteworthy that Evans et al. (12) reported differences of up to 6 weeks in the time required for restoration of fertility in individual animals by linoleic acid supplementation.

The results reported in this paper again emphasize the differences between tissues in their ability to incorporate unsaturated fatty acids. Of the 3 tissues considered, heart incorporated the ω6-series acids and lost ω9-acids most readily. In this respect it closely resembled plasma and liver (3). Heart lipids also incorporated linoleic acid more readily than arachidonic acid, thus exhibiting a fatty acid specificity similar to plasma, erythrocytes and liver. Brain lipids incorporated the ω6-acids more slowly than most other tissues studied and no preferential incorporation of linoleic acid was observed.

Rat testes differed from all the other tissues studied. The overall changes were slow. Elevated levels of palmitoleic and oleic acids persisted for practically the entire period of the experiment and linoleic acid was incorporated relatively slowly. This tissue accumulated arachidonic acid more readily than linoleic acid, which was in marked contrast with the other tissues studied with the exception of brain which is normally low in linoleic acid. The rapid accumulation of arachidonic acid may reflect a metabolic block in the unsaturated acid elongation-desaturation pathway between arachidonic acid and 22:5ω6, resulting from structural disruption of particulate enzyme systems brought about by essential fatty acid deficiency. This possibility bears further investigation.

As in our previous studies, recovery of brain, heart and testes lipids from a state of essential fatty acid deficiency was found to follow a course which was specific for the tissue under consideration and for particular fatty acids.

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Serum Cholesterol Levels in Rats Fed Thirteen Trace Elements [1]

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ABSTRACT To ascertain differences in serum cholesterol levels as possibly affected by trace elements in the drinking water, rats were given soluble salts of zirconium, vanadium, niobium, chromium, nickel, cadmium, germanium, tin, lead, arsenic, and antimony at 5 ppm and selenium and tellurium at 2 ppm from the time of weaning until 11 to 30 months of age. The feeding of chromium at 1 ppm was associated with suppressed levels in males but not in females; 5 ppm appeared to be required for this effect in females. The lowest serum cholesterol levels were observed in groups given niobium, chromium and nickel, the highest in groups fed tellurium, with all animals receiving 1 ppm chromium. Significant differences in values of the 2 sexes appeared for 7 elements. It is possible that chromium, nickel and niobium exert anti-cholesterogenic properties, whereas tellurium may be cholesterogenic.

Mean circulating cholesterol levels in human beings tend to rise with age. This tendency is exaggerated in persons living in industrialized countries and is less evident in more primitive societies. The possibility arose that some abnormal trace element to which civilized man is exposed and which accumulates in his tissues with age might influence the synthesis of cholesterol or interfere with its catabolism. Conversely, some modern nutritional practices might promote marginal deficiency of an essential trace element on which homeostasis of cholesterol depends.

Curran (1) showed that rat liver, in vitro and in vivo, synthesized cholesterol less well in the presence of vanadyl ions and better in the presence of trivalent chromium and manganous ions than liver unexposed to trace elements. Other trace metals in the first transitional series were relatively inert. Curran's rats were fed a commercial ration, which at that time was marginally deficient in chromium (2). The action of chromium feeding in stabilizing serum cholesterol at a low level in male rats has been demonstrated (3, 4); associated with this effect was virtual abolition of spontaneous aortic plaques and accumulation of aortic lipids (4).

Studies on the life-term effects of a number of trace elements given in low doses to rats and mice (5) have provided an opportunity to measure serum cholesterol and glucose levels. The present report concerns 13 trace elements fed to rats from the time of weaning. Differences in serum cholesterol associated with element and age are shown.

METHODS

The environmental conditions in the laboratory, the precautions taken to avoid metallic contamination, the low metal diet and the double-deionized drinking water used have been reported in detail (6, 7). The trace element content of the diet, which was composed of seed rye, powdered skim milk and corn oil, and the estimated total intakes, are shown in table 1. To the basic drinking water, which contained zinc (50 ppm), manganese (10 ppm), copper (5 ppm), cobalt (1 ppm), molybdenum (1 ppm) and chromium (1 ppm), was added one of the following at 5 ppm element; zirconium sulfate, sodium niobate, antimony potassium tartrate, sodium germanate, stannous chloride, sodium arsenite, vanadyl sulfate, nickelous acetate, cadmium acetate, lead acetate. Sodium

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TABLE 1

Trace element content of diet and drinking water and estimated daily intakes [1,2]

	Diet	Water	Total
	µg/g	*µg/g*	*µg/100 g body wt/day*
Zirconium	2.66	5	50.96
Vanadium	3.24	5	54.44
Niobium	1.62	5	44.72
Chromium	0.16	0	0.96
Chromium	0.16	1	7.96
Chromium	0.16	5	35.96
Nickel	0.44	5	37.64
Cadmium	0.14	5	35.70
Germanium	0.32	5	36.92
Tin	0.28	5	36.68
Lead	0.20	5	36.20
Arsenic	0.46	5	37.76
Antimony	nd [3]	5	35.0
Selenium	0.05	2	14.30
Tellurium	0.16	2	14.96

[1] Based on 6 g food and 7 g water ingested/100 g body weight/day.
[2] These intakes are approximate but are comparable. Gains in weight of young rats were similar, weights of adult animals remained fairly constant, and variations among groups were not observed. The intake of water was measured on control rats for one year; marked variations in other groups were not evident.
[3] Not detected.

selenate and sodium tellurite were added at 2 ppm element; sodium selenite at 3 ppm.[3] In addition, the basic water was given without chromium or with 5 ppm as the acetate.

Random-bred pregnant female rats were purchased from a supplier [4] and their offspring weaned at 21 to 23 days. Groups of 52 or more of each sex, four to a cage, were given one of the trace elements until natural death occurred. Each group was placed in separate wooden racks, and cages were not interchanged between groups.

In addition, 4 generations of rats were bred without exposure to chromium other than the small amount in the diet, as reported (8). The second and fourth generations were used in these experiments. Thirty brood females obtained from the supplier, which had been fed a diet containing adequate chromium, were also studied. Their offspring were not believed to be deficient in chromium according to analyses of tissues of similarly bred animals (9).

Blood was obtained from the tail of the warmed rat by cutting with a razor blade, and centrifuged. Serum cholesterol was measured by the method of Huang et al. (10), using a premixed reagent and a Berkeley Medical Instrument spectrophotometer;[5] a few were measured by the method of Abell et al. (11). Duplicate and replicate analyses agreed within 3%.

Analyses of the diet for trace elements were made by a number of standard methods, colorimetric for zirconium, vanadium, niobium, chromium, nickel, germanium, arsenic, tin, lead and selenium, and atomic absorption spectrophotometric for chromium, cadmium, antimony and tellurium.

RESULTS

Serum cholesterol levels in rats given various amounts of chromium are shown in table 2. Sex differences appeared. Males fed 1.0 to 5.0 ppm in drinking water had lower mean values than those given none in water. Females given 5.0 ppm in water or about 2 ppm in food and water had lower values than those given 1.0 ppm or less. The difference in male and female values at 1.0 ppm was significant ($P < 0.001$). According to these data, female rats apparently require more chromium than males in order that cholesterol in serum be suppressed. Fourth generation chromium-deficient young rats of both sexes had higher values than second generation young rats which were assumed to be less deficient.

In table 3 are the mean serum cholesterol levels of rats fed 13 trace elements in drinking water, arranged according to the values of males. Again sex differences appeared in 7 cases. Male values were higher in the groups given vanadium, chromium, selenium ($P < 0.025$), and in the controls without chromium ($P < 0.05$). Female values were higher in those given cadmium ($P < 0.05$), germanium and tin ($P < 0.001$). More or less consistently low values were noted in the groups of both

[3] In a previous publication on the effects of selenite and selenate on mice and rats (14), the concentration of selenium in the selenite given in water was erroneously reported as 2 ppm. The supplier of sodium selenite (Nutritional Biochemicals Corporation, Cleveland) stated in a letter that this product was $Na_2SeO_3 \cdot 5H_2O$. Nearly 2 years later the compound was found by D. V. Frost, in our laboratory, to be anhydrous, and was then so admitted to be by the supplier. As the hydrated form contains 45.7% selenium and the hydrated form 30%, the rats and mice given selenite actually received 3 ppm selenium.
[4] Blue Spruce Farms, Altamont, New York.
[5] Berkeley Medical Instruments, New England X-Ray and Electronic Equipment, Brookline, Massachusetts 02146.

TABLE 2

Serum cholesterol levels in rats given chromium

	Chromium	Males [1]			Females [1]		
		No.	Age		No.	Age	
	ppm		*days*	*mg/100 ml*		*days*	*mg/100 ml*
Young [2]							
F_2	0	11	204	86.0±3.9 [3]	12	204	78.5± 2.5
F_4	0	8	115	114.0±5.0 [4]	10	127	109.6± 2.7 [4]
Mature							
	0	12	761	122.9±8.2	12	761	94.5±11.2
	1	23	662	77.5±2.1 [5]	19	660	116.0± 6.0
					7	689	108.6± 4.2
	2±				30	250 [6]	83.3± 1.7 [7]
	5	12	405	86.2±3.2 [5]	12	405	72.1± 5.3 [8]
	5	12	510	77.0±6.6 [5]	8	709	63.0± 4.2 [9]

[1] Male and female values differ significantly: 2.0 ppm group, $P < 0.025$; 1.0 ppm group, $P < 0.001$; 5.0 ppm groups, $P < 0.025$.
[2] F_2 and F_4 are rats bred from increasingly deficient mothers.
[3] SE.
[4] Differs from F_2 group, $P < 0.001$.
[5] Differs from mature group, no chromium, $P < 0.001$.
[6] Breeders purchased while pregnant, food containing chromium.
[7] Differs from mature groups, 1.0 ppm 660 and 689 days old, $P < 0.001$.
[8] Differs from 2.0 ppm group, $P < 0.01$.
[9] Differs from 2.0 ppm group, $P < 0.001$.

TABLE 3

Serum cholesterol levels in mature rats given various trace elements

	Males			P value [1]	Females			P value [1]
	No.	Age			No.	Age		
		days	*mg/100 ml*			*days*	*mg/100 ml*	
Germanium	12	557	63.4± 3.2 [2]	< 0.001	12	578	107.6± 5.9	ns [3]
Nickel	12	342	75.3± 2.6	ns	12	329	75.3± 3.8	< 0.001
Niobium	12	849	75.7± 2.7	ns	11	849	78.6± 4.8	< 0.001
Controls (Cr 1)	23	662	77.5± 2.1	—	19	660	116.0± 6.0	—
Tin	12	520	82.5± 3.4	ns	12	580	105.3± 3.5	ns
Chromium	12	405	86.2± 3.2	< 0.02	12	405	72.1± 5.3	< 0.001
Lead	14	695	86.6± 6.5	ns	12	760	103.7± 8.5 [4]	ns
Cadmium	10	750	88.6± 8.7 [4]	< 0.05	12	750	113.0± 9.0 [4]	ns
Zirconium	12	893	89.7± 5.6	< 0.01	12	893	100.7± 9.0	ns
Arsenic	12	804	91.0±10.1	< 0.05	13	806	109.5± 7.4	ns
Vanadium	11	761	91.6± 5.1	< 0.005	10	761	67.9± 9.2	< 0.001
Antimony	10	790	97.6± 4.9	< 0.001	10	790	97.0± 5.6	< 0.01
Selenium (VI)	12	445	109.6± 6.1	< 0.001	12	445	90.4± 5.0	< 0.005
Selenium (IV)					12	445	90.2± 3.5	< 0.005
Tellurium	12	405	111.4± 3.0	< 0.001	12	452	110.4± 9.9	ns
Controls (no Cr)	12	761	122.9± 8.17 [4]	< 0.001	12	761	94.5±11.19 [4]	< 0.05

[1] Significance of differences from controls.
[2] SE.
[3] Not significant.
[4] Method of Abell et al. (11), previously reported (4), for comparison.

sexes given niobium, chromium, and nickel, and high values in those fed tellurium.

Compared with the controls given 1.0 ppm chromium, values for males were especially elevated in groups fed tellurium, selenium, antimony and no chromium and slightly elevated in the groups fed zirconium and vanadium. Cholesterol levels in males given germanium were suppressed.

Female control levels were the highest of any group. Other elevated values (> 100 mg/100 ml) occurred in the groups given zirconium, cadmium, germanium, tin, lead, arsenic and tellurium. Low values (< 80 mg/100 ml) were found in the groups given vanadium, niobium, chromium and nickel.

Because two of these elements, chromium and vanadium, have been shown to

affect cholesterol metabolism in vitro and in vivo (1, 4), comparisons were made as to significant differences in circulating cholesterol between rats fed these metals and other elements (table 4). In relation to the chromium-fed group, levels were higher in male rats given antimony, selenium and tellurium and lower in those fed niobium, nickel and germanium. In females, mean values were higher in rats given all of the elements except niobium and nickel.

Comparisons of levels of male rats fed vanadium with those given other elements showed significantly elevated values only in rats fed selenium and tellurium. Significantly depressed values were observed in those given niobium, nickel and germanium. In females, all values, except those

TABLE 4

Significance of differences in serum cholesterol levels of rats given various trace elements compared with those given chromium and vanadium

	Males				Females			
	Chromium		Vanadium		Chromium		Vanadium	
	Difference [1]	P value [2]	Difference [1]	P value [2]	Difference [1]	P value [2]	Difference [1]	P value [2]
Controls, no Cr	+	< 0.001	+	< 0.001	+	< 0.05	+	< 0.05
Controls, 1 ppm Cr	—	< 0.02	—	< 0.005	+	< 0.001	+	< 0.001
Zirconium		ns [3]		ns	+	< 0.01	+	< 0.01
Vanadium		ns				ns		
Niobium	—	< 0.01	—	< 0.005		ns		ns
Nickel	—	< 0.01	—	< 0.005		ns		ns
Cadmium		ns		ns	+	< 0.001	+	< 0.001
Germanium	—	< 0.001	—	< 0.001	+	< 0.001	+	< 0.001
Tin	—	ns		ns	+	< 0.001	+	< 0.001
Lead		ns		ns	+	< 0.001	+	< 0.005
Arsenic		ns		ns	+	< 0.001	+	< 0.001
Antimony	+	< 0.02		ns	+	< 0.01	+	< 0.02
Selenium	+	< 0.001	+	< 0.001	+	< 0.01	+	< 0.02
Tellurium	+	< 0.005	+	< 0.005	+	< 0.001	+	< 0.005

[1] Plus sign indicates that the value is larger; minus sign, smaller than that for chromium or vanadium.
[2] Significance of difference in the value from that of chromium or vanadium (see table 2).
[3] Not significant.

TABLE 5

Changes with age in serum cholesterol in rats fed zirconium, niobium, antimony, lead and cadmium

Element	Males			P value [1]	Females			P value [1]
	No.	Age			No.	Age		
		days	*mg/100 ml*			*days*	*mg/100 ml*	
Zirconium	12	270	69.0 ± 3.8 [2]		12	270	76.0 ± 1.8	
	12	893	89.7 ± 5.9	< 0.005	12	893	100.7 ± 9.0	< 0.01
Niobium	12	166	69.3 ± 3.4		12	228	81.1 ± 3.7	
	10	655	70.3 ± 2.89	ns [3]				
	12	849	75.7 ± 2.7	ns	11	849	78.6 ± 4.8	ns
Antimony	12	125	76.7 ± 2.4		12	161	86.3 ± 4.4	
	10	790	97.6 ± 4.9	< 0.001	10	790	92.6 ± 5.6	ns
Lead	11	300	72.3 ± 4.74					
	12	510	79.3 ± 6.48	ns	12	510	103.4 ± 7.12	
	14	695	86.6 ± 6.7	ns				
	12	750	74.0 ± 10.03	ns	12	750	103.7 ± 8.53	ns
Cadmium	10	300	76.1 ± 4.74					
	10	485	85.6 ± 2.3	< 0.05	10	485	97.0 ± 8.9	
	12	510	67.6 ± 2.81	ns	12	510	86.5 ± 9.80	ns
	12	750	88.6 ± 8.70	ns	12	750	113.0 ± 8.98	ns
Arsenic [4]	12	770	91.6 ± 10.5					
	12	804	91.0 ± 10.1	ns				

[1] Significance of difference from first value in each group.
[2] SE.
[3] Not significant.
[4] For comparison of repetitive analyses on different rats of same group.

of animals given niobium and nickel, were significantly elevated.

Changes in circulating cholesterol levels with age are shown in table 5. No increase was found in animals fed niobium, lead or cadmium, nor in females given antimony. Increases occurred in rats of both sexes fed zirconium and in males fed antimony. Data on chromium (table 1) show some decrease with age, especially in females.

DISCUSSION

The rat is a poor animal for the study of experimental hypercholesteremia, requiring special diets and the feeding of cholesterol and saturated fats to develop high serum levels. The present study concerns adequate diets and unsaturated fats (corn oil), and high serum levels were only noted occasionally. Nevertheless, trends and tendencies appeared which might turn out to be significant in more susceptible mammals.

On this basis, these data indicate that trivalent chromium given to rats in drinking water was associated with lowered serum cholesterol levels, as shown previously (4), that chromium deficiency in young rats was associated with elevated levels, and that female rats appeared to require larger doses for this effect to appear than males. The data show that, in general, higher levels occurred in male rats fed the metalloid or nonmetallic elements in the "A" groups of the Periodic Table — arsenic, antimony, selenium and tellurium — than in those fed metals in the transitional or "B" groups — vanadium, niobium, nickel and cadmium — or heavy metals in the "A" groups — tin and lead. Female rats, however, also had higher levels when they were given those elements situated in the right of the Periodic Table, beginning with cadmium, and lower levels when fed elements situated in the left or "B" groups.

To test this impression, the elements were arranged in order of position in the Periodic Table from left to right and assigned numbers from 2 (zirconium) to 27 (tellurium). Paired rank correlations with serum cholesterol levels were calculated. For males, $r = +0.486$ ($P \sim 0.05$); for females, $r = +0.622$ ($P < 0.01$). Although the list of elements studied is incomplete, those on the right of the Table were more likely to be associated with high serum cholesterol levels than were the transitional metals on the left. There was no significant correlation with atomic number. In the experience of this laboratory, which will be reported, the elements on the right of the Table were more likely to exert innate or overt toxicity in mice and rats than were the transitional metals.

The data on changes in serum cholesterol with age are incomplete. They suggest, however, that age-linked increases can occur in marginal chromium deficiency, in rats of both sexes fed zirconium and in males fed antimony. They suggest that such increases may not occur in rats given niobium, lead and cadmium.

When the groups fed tellurium were compared with the others, as in table 3, significant differences appeared largely in males. Probabilities (P) of the differences being due to chance of the order of <0.005 occurred in the zirconium, niobium, chromium, nickel, germanium, tin, lead and control groups, of < 0.01 in the cadmium group, of < 0.025 in the antimony group and < 0.05 in the arsenic group, all values being smaller. Only the selenium group did not so differ. In females, the vanadium, niobium, chromium, and nickel groups differed ($P < 0.005$), as possibly did the selenium group ($P < 0.05$), values being smaller than those of the tellurium group.

Because it was possible that differences in cholesterol levels might be related to factors other than the trace elements fed to the animals, attempts were made to avoid unknown influences as far as practicable. Males and females were strictly comparable. Several groups of animals were bred and observed simultaneously as follows: Controls (no chromium), chromium (5 ppm), cadmium and lead; contrals (1 ppm chromium), arsenic, germanium and tin; lead (males), zirconium, niobium and antimony; chromium (5 ppm), vanadium, nickel, selenium and tellurium. Each experiment required 4 years for completion (5). As facilities were limited to 1,000 rats, additional groups were bred and started, usually in the fall or winter, when previous groups were partly depleted through death. Analyses

of sera were made during the summers on several groups at weekly intervals. The diet was as uniform as feasible, although annual variations in the trace element content of seed rye obtained from the same area each year were possible, owing to annual variations in rainfall. No consistent changes were found among the groups analyzed at each interval which could be attributed to unknown factors.

The values obtained by the method of Abell et al. (11) in the first series (4) were compared with those obtained by that of Huang et al. (10) which was an essentially similar but more simple method. Mature males fed lead in the first series (4) had 79.3 ± 6.5 mg cholesterol/100 ml; those in the second had 86.6 ± 6.5 mg /100 ml. Mature males fed chromium had 77.0 ± 6.6 mg/100 ml in the first series and 86.2 ± 3.2 mg/100 ml in the second. Apparently the 2 methods gave values in the same ranges for 2 sets of animals fed the same metals.

Because all these animals, except those in one control group, were given chromium, it is evident that if an element influenced serum cholesterol levels, the effect was either additive to or antagonistic to that of chromium. Additive effects in one sex might be postulated for niobium and nickel; antagonistic effects for tellurium, selenium, antimony, vanadium and zirconium.

Therefore, it is possible that a common property of nickel, niobium and chromium may be to influence serum cholesterol of rats at low levels, and that tellurium in some way may raise it. The lowest standard deviations, and therefore individual variations, were found among analyses of sera from rats fed these metals. Tissue levels of the various elements will be or have been reported (5). Application of these data to the chromium deficiency noted in samples from the adult population of the United States (9) and the excess of tellurium (12, 13) deserve study.

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Factors Influencing Urinary Taurine Excretion by Normal and Mongoloid Subjects [1]

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ABSTRACT The effect of a variety of experimental conditions on urinary taurine excretion by normal and mongoloid subjects was examined. Measurement of taurine was by column chromatography. Habitually "high" and "low" excretors were demonstrably present in both populations. In normal subjects taurine excretion was increased by the ingestion of sulfur amino acid-rich protein, shellfish, cysteamine, hypotaurine, or prednisone; cysteine sulfinic acid and vitamin B_6 had no effect; cysteic acid and cysteine had little, if any, effect. While under a taurine load of 4 mg/kg body weight/day, a roughly 3-day periodicity in taurine excretion was noted in both normal and mongoloid subjects. Six weeks of vitamin B_6 administration did not correct the subnormal excretion of taurine by mongoloids. Renal tubular reabsorption of taurine appears to be greater in mongoloid low excretors than in high excretors. No correlation between serum and urine taurine values was found. Fasting serum taurine levels varied more widely than had been expected, both intra- and inter-individual, and there is a tendency toward bimodality of these data.

Of the free amino acids appearing in normal adult urine, taurine is second both in amount (13% of total) and variation (27 to 230 mg/day range) (1). There is increasing evidence to suggest a metabolic role for taurine in higher animals (2, 3), particularly in function of nervous tissue (4, 5). Thus it appeared useful to examine further the factors affecting urinary taurine excretion. Some influences established with varying degrees of certainty are age (6), pregnancy (7), certain foodstuffs (8), vitamin B_6 intake (9–13), intestinal flora (10, 14), irradiation (15, 16), thermal burns (17, 18), and certain other forms of stress (19, 20). Our interest in this problem arose from observation of occasional unexplained increases in taurine excretion by mongoloid subjects who generally had subnormal excretion (21); consequently, such individuals are the subjects for several of the experiments reported here.

SUBJECTS AND METHODS

Subjects for experiments to be described included colleagues and medical students in apparent good health and mongoloid and non-mongoloid retardates residing at Western Carolina Center (W.C.C.), Morganton, North Carolina, or Caswell Center (C.C.), Kinston, North Carolina. All mongoloids at W.C.C. were karyotyped and were found to have the standard trisomy 21. All mongoloid subjects at C.C. were examined by two of the authors and any for whom the diagnosis was considered equivocal were excluded.

In all experiments involving retardates at W.C.C. or C. C., mongoloids were matched with non-mongoloid subjects for sex, weight within 2.27 kg and age within 2 years. All retarded subjects were receiving the same institutional diet. Even if a subject was fed a soft diet, the constituents of the regular diet were simply ground and fed. Observations of subjects during several meals made it apparent that virtually all subjects consumed all food provided. It is, of course, an assumption that whatever food preferences exist average out between groups of mongoloid and non-mongoloid subjects, though little evidence

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of strong food preferences was observed by us or reported to us by dietitians at the 2 institutions.

Taurine was determined in urine and serum by column chromatography (21). Homogeneity of the taurine peak was shown by its consistent symmetry, the identity of the ratio of the peak areas measured at 440 mμ and 570 mμ with that of taurine, and paper chromatographic examination of the appropriate fraction of the eluate. Creatinine was determined by the method of Taussky (22). Creatinine was used as a base for taurine excretion because it provides values most nearly approximating 24-hour taurine excretion values for specimens collected during shorter time-periods. Further, this base removes the association between taurine excretion and both weight and age (21).

RESULTS AND DISCUSSION

To examine the effect of protein intake on urinary taurine levels, 6 normal adults (5 males, 1 female) consumed identical diets for 5 days. On each day, taurine was measured a) in the first morning urine, b) in that collected between arising and breakfast (which we and others (23–25) find most nearly corresponds to the complete 24-hour urine in terms of creatinine/hour), and c) in that collected during the remaining 15 to 15.5 hours. On days 1 and 2 the daily protein intake was 20 g, on days 3 and 4 it was 120 g and on day 5 it was 80 g. The diets were approximately isocaloric. Urine specimens were collected for 6 days including the day following the 80-g protein intake. The first morning and pre-breakfast specimens on the first day were the only control specimens collected. Our purpose in this experiment was to measure the maximal fluctuations that might be expected due to short-term dietary manipulation as an aid to interpreting the fluctuations observed from time to time in specimens from a single retarded subject (for example, 20 and 80 mg taurine/g creatinine on two consecutive days). Further, the few previous studies of the influence of diet on taurine excretion were based on paper chromatographic analysis with its lower resolving power and difficulties in quantitation. The chosen levels of protein intake far exceed variations found in the normal institutional diet. The measurements on the pre-breakfast specimens of urine for the 2 highest and the 2 lowest excretors are presented in figure 1 and show the clear differences in individual response.

The results for the 15-hour urine specimens collected from all subjects during the day and evening are presented in table 1. An analysis of variance of these data demonstrated a highly significant effect of both diet and individual differences. The means suggest that effects of a low protein diet are not completely manifested on the first day. Similarly, the effect of high protein intake appears to carry over to the following day. Analyses were also performed on both the first morning urine specimens and on those collected after the first morning specimen but before breakfast (0.5 to 1 hour). The results of these analyses were comparable to those cited above except that the proportion of variability attributable to individual differences was greater than that attributable to diet in the latter 2 analyses. The first morning specimens were the least power-

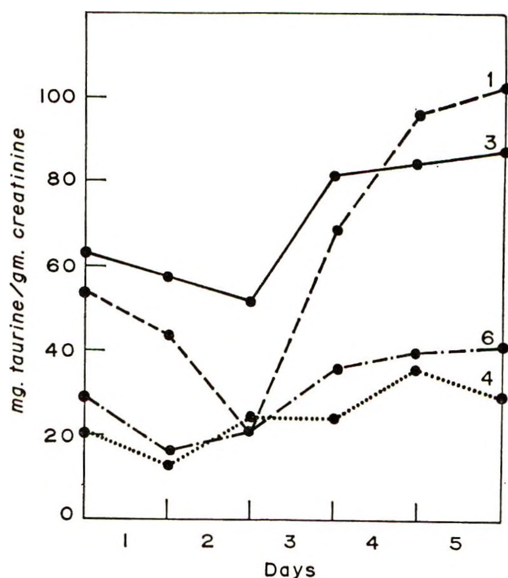

Fig. 1 Taurine content of urine samples collected from subjects 1, 3, 6 and 4 after morning voiding but before breakfast with low protein (days 1 and 2), high protein (days 3 and 4) and normal protein (day 5) diets.

TABLE 1

Taurine values of 15-hour urine specimens from 6 apparently normal adult subjects, collected during low protein, high protein and normal protein dietary regimens

	Days	Subject no.						Means
		1	2	3	4	5	6	
		mg taurine/g creatinine						
Low protein	1	59	48	62	50	85	33	56.2
	2	60	24	40	44	69	29	44.3
High protein	3	83	50	87	39	62	58	63.2
	4	116	87	123	79	109	72	96.8
Normal protein	5	111	40	116	73	67	40	74.5
Means		85.8	49.8	85.6	56.0	78.4	46.4	
Rank		1	5	2	4	3	6	

Analysis of variance: Variance between days relative to error, $F = 10.95$, $P < 0.001$; between individuals relative to error variance, $F = 7.67$, $P < 0.001$.

ful detectors of either individual or dietary differences, as might be expected from the generalized slowing of most metabolic activities during the night. These data also suggest that these normal subjects can be divided into at least 2 groups, subjects 1 and 3 having higher means than all others in all 3 sets of specimens. These 2 subjects have consistently had higher values than the remaining subjects both in all experiments reported here and in a variety of other unpublished studies.

One of the "high excretors" and one of the "low excretors" from the preceding experiment had their urinary taurine measured on 3 successive days after either a sulfur amino acid-rich breakfast (4 eggs, toast and coffee), breakfast plus 50 mg L-cysteine/kg body weight, or breakfast plus 4 mg taurine/kg body weight. The response again demonstrated individual differences (table 2).

Two of the high excretors (1 and 3 from table 1) and one of the low excretors (subject 6 from table 1) ingested 50 mg L-cysteine/kg body weight, and taurine excretion was measured at intervals during periods up to 29 hours, with no alteration in normal diet or routine. The same procedure was carried out with two low excretor monogoloids, for comparison (table 3). The excretion of taurine was increased in each case during the succeeding day, the increase representing a cysteine-to-taurine conversion of approximately 1 to 2% in the normal individuals (13), but less in the mongoloids. An anomalous exception is the excretion of taurine corresponding to 1.4% of the ingested cysteine by one of the mongoloids in a 2-hour period.

TABLE 2

Urinary excretion of taurine of 2 individuals after 3 dietary regimens

	Hours	Taurine	
		Low excretor	High excretor
		mg/g creatinine	
Breakfast	0	31	104
	2	39	100
	4	32	—
	6	39	83
Breakfast + cysteine	0	25	86
	2 [1]	26	107
	4	31	157
	6	68	160
	10	—	157
Breakfast + taurine	0	14	93
	2 [2]	280	780
	4	309	456
	6	164	218

[1] Serum taurine: 128 and 66 μmoles/liter, respectively.

[2] Serum taurine: 171 and 104 μmoles/liter, respectively.

Since the subnormal excretion of taurine by mongoloids appeared to be the result of a tissue deficit of taurine (26), it was of interest to compare the effect of a daily load (4 mg/kg body weight) of taurine on taurine excretion, as well as the superimposed intake of either shellfish (8), metabolic precursors of taurine, or prednisone.

TABLE 3
Urinary taurine response to cysteine loading

Time after dose	Subject designation				
	Normal			Mongoloid	
	3	1	6	AA	AL
hours	*mg taurine/g creatinine*				
0	68	55	32	10	15
2	76	52	32	7	22
4	114	90	58		22
6	160	58			20
8	155	68	66	13	23
10	131	116		28	63
12	88	discarded		34	930
14		141	58		
16				19	22
18					
20	105			15	23
24	111	66	51		
29		100			

Figure 2 shows the results with such a study of 4 normal and 3 mongoloid subjects.

There was a rather striking rhythmicity in the taurine excretion, even if the diet was held constant; all subjects showed an irregular but roughly 3-day periodicity. Under these conditions, the three or four days required for excretion to plateau suggested that the tissues are not normally saturated with taurine. If the normal excretion and the equilibrium excretion be used to estimate roughly the degree of undersaturation, it amounts to 149 mg in normal subject 1 and 1154 mg in mongoloid AA. In neither case is this very impressive in comparison with the total body taurine, which can be very roughly estimated at 18 g (3, 27, 28) although the "exchangeable" taurine may be much less (15, 16, 27). Prompt elevations in taurine excretions were produced in normal subjects by ingestion of cysteamine, hypotaurine, taurine, prednisone, or shellfish. Cysteine sulfinic acid, vitamin B_6, $NaHCO_3$, or 177 ml of alcohol had no effect. Cysteic acid and cysteine had doubtful effect (29). Table 4 shows the taurine excretion (morning specimens) of 6 mongoloids after being given such a taurine load for several weeks. All were excreting less than the normal individuals when they were in equilibrium with this load.

This suggested that such loading might be useful in separating the mongoloids more distinctly into excretor classes (21). However, measurement of urinary taurine after 4 days of loading (when equilibrium values would have been attained) gave the results shown in figure 3, with no indicator of class-segregation.

In a previous paper (30), results were reported which encouraged the belief that the low taurine excretion observed in most mongoloids could be corrected by the administration of vitamin B_6. Indirect support for this belief was provided by McCoy et al. (31), who reported that some mongoloids were slightly deficient in vitamin B_6 as evidenced by tryptophan loading, and by other studies (9–13). The administration of deoxypyridoxine gave results suggesting a more complicated situation (32). To examine this possibility more thoroughly, 50 mongoloid subjects at C.C. were divided into a treatment and a control group; the treated subjects received 50 mg vitamin B_6/day[4] for 6 weeks, the control subjects, neither drug nor placebo (one mongoloid from the treatment group left the institution during the course of the study). A baseline early morning urine specimen was obtained initially and early morning specimens were collected after 2 and 6 weeks. Taurine was measured in all specimens and the results normalized by conversion to $\log_{10}$ (mg taurine/g creatinine). Table 5 presents descriptive statistics calculated from these data.

The grand mean (1.30) for these data does not differ significantly from the mean of 1.13 previously obtained (21) for 127 mongoloid subjects and is significantly lower than the mean of 1.68 found then for control subjects. Paired *t* tests of values for treated mongoloid subjects reveal that those at 2 weeks were significantly lower than either baseline or 6-week values ($t = 3.21$, $P < 0.01$ and $t = 3.80$, $P < 0.001$, respectively). Similarly both baseline and 2-week values were significantly lower than 6-week data for control subjects ($t = 2.90$, $P < 0.02$ and $t = 5.01$, $P < 0.001$, respectively). By inspection it appears that the only possibly significant difference between corresponding means for treated and untreated subjects is that between baseline means; a group test of those means ap-

[4] Kindly supplied, as Hexa-Betalin tablets, by the Lilly Research Laboratories, Indianapolis, Indiana.

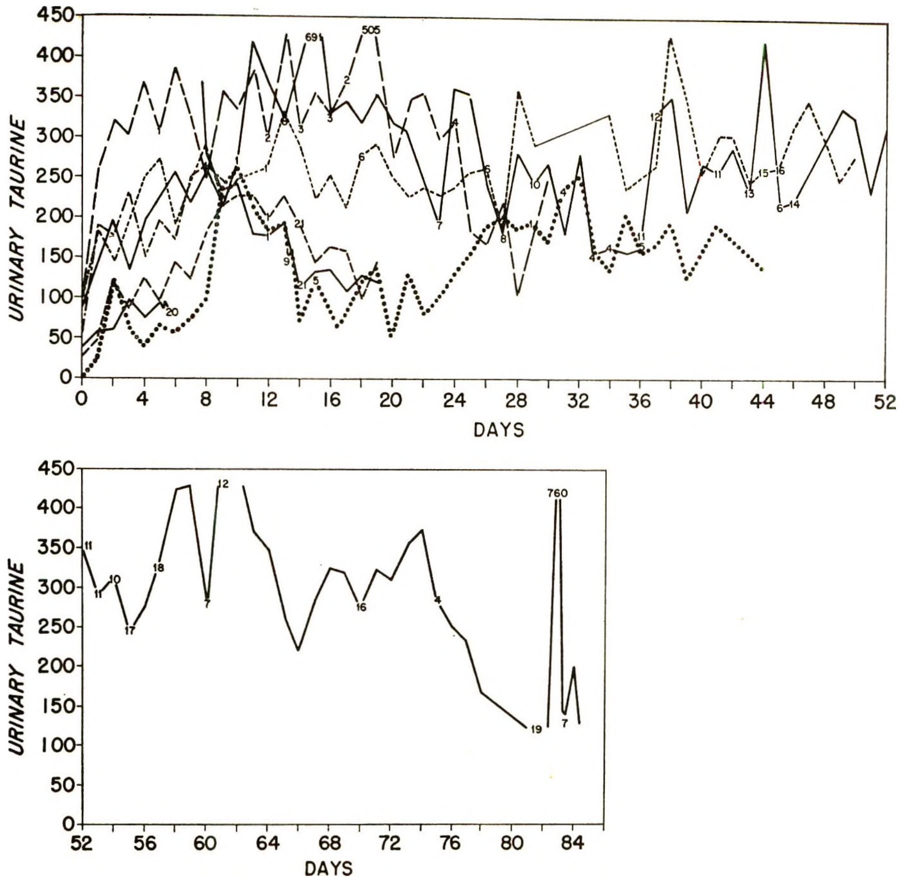

Fig. 2 Response of urinary taurine (mg/24 hours) to 140 mg taurine twice a day for an extended period. The numerals denote that in the succeeding 24-hour period the following substances were ingested or the following modifications made: 1) 3.5 g cysteine; 2) boiled clams; 3) constant diet; 4) off taurine; 5) taurine resumed; 6) 1 g cysteine sulfinic acid; 7) boiled shrimp; 8) 500 mg hypotaurine; 9) off taurine, 8 days with control determination following; 10) 5 g $NaHCO_3$; 11) 50 mg vitamin B_6; 12) 1 g cysteamine; 13) 20 mg prednisone; 14) 5 g cysteine; 15) 177 ml ethanol; 16) 2 g cysteic acid; 17) 140 mg extra taurine; 18) 280 mg extra taurine; 19) 71 g raw clams; 20) dental surgery with general anesthesia; and 21) 10 eggs in diet. Subjects were 4 normal individuals (upper curves) and 3 mongoloid adults (3 lower curves).

TABLE 4

Taurine excretion by mongoloids given 4 mg taurine/kg/day for 5 weeks

Subject no.	Before load	After load
	mg taurine/g creatinine	
1	15	183
2	16	182
3	22	173
4		145
5	20	200 [1]
6	4	86 [1]

[1] Taurine load given for 3 weeks.

proached but did not reach conventional significance levels ($t = 1.91$, $P > 0.05$), illustrating the greater power of paired comparisons to detect differences when it is logical and feasible to make such comparisons.

These data do not support the previous impression that vitamin B_6 can affect urinary taurine excretion among mongoloids. However, they document the fact that urinary taurine excretion may shift significantly from one time to another in the same subjects. The previous experiment

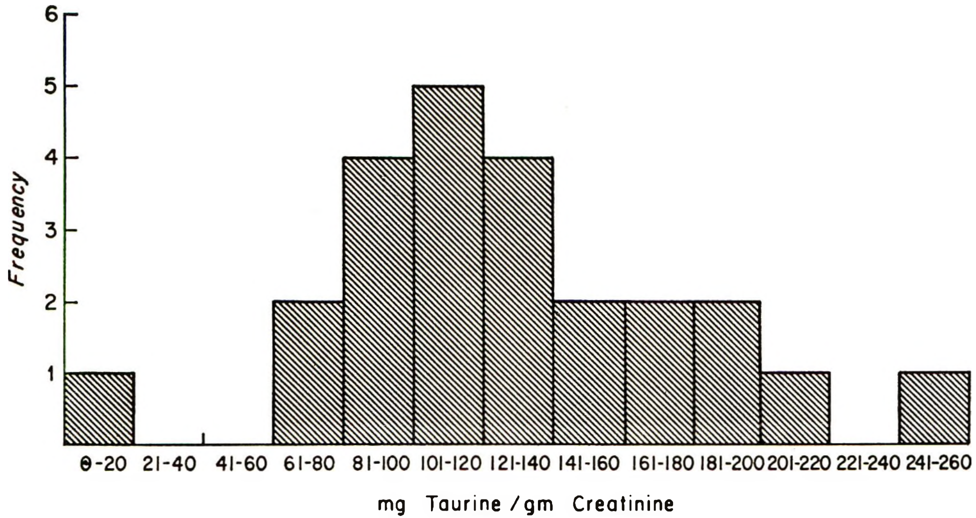

Fig. 3 Distribution of taurine content of morning specimens of urine from 24 mongoloids after 4 days of ingesting 4 mg taurine/kg body weight/day.

TABLE 5

Baseline, 2-week and 6-week urinary taurine excretion among mongoloid subjects treated with 50 mg vitamin B_6 for 6 weeks and among untreated mongoloid controls

Subjects	No.	Baseline	2-weeks	6-weeks	Total
			log_{10} (mg taurine/g creatinine)		
Treated	24	1.48 ± 0.11 [1]	1.14 ± 0.08	1.49 ± 0.08	1.37 ± 0.06
Untreated	25	1.16 ± 0.12	1.11 ± 0.08	1.56 ± 0.07	1.28 ± 0.06

[1] SE of mean.

supports the conjecture that diet may be responsible for most of this variability and the fair correspondence lends support, the more so since the same food was served to all subjects, individual preferences being a potential source of uncontrolled variation.

In previous work, taurine had been measured in a few sera from mongoloids and values found were within the normal range as determined in this laboratory (88.6 ± 30.8 μmoles/liter) (30) and elsewhere (33). Subsequently 4-hour clearance studies were performed on 23 mongoloid subjects and a few non-mongoloid retardates. The lowest mongoloid excretors generally were found to resorb 98 to 99% of the taurine reaching the kidney, whereas the intermediate mongoloid excretors and most non-mongoloid retardates resorbed between 86 and 97%, the normal percentage range (33). No correlation was observed between these serum and urine taurine values ($r_{xy} = 0.02$, $P > 0.50$).

Serum taurine levels, like those of urine, can exhibit marked variations from one time to another, even in fasting subjects as illustrated by data in table 6. These matched subjects were studied 3 weeks apart following overnight fasting. The mongoloid subjects had significantly higher values than their retardate controls in both the first and second collection ($t = 3.66$, $P < 0.02$ and $t = 3.34$, $P < 0.02$, respectively). The correlation between times was large but not significant, presumably because of small sample sizes ($r_{xy} = 0.66$, $0.10 > P > 0.05$). These results encouraged belief that the lower mean urinary excretion of taurine among mongoloids might be reflected in higher serum taurine levels. To test the generality of this inference, we tested all the mongoloids available at W.C.C.

Each monogoloid was matched with a non-mongoloid retardate, but it was not feasible to collect all specimens on the

TABLE 6

Two determinations of serum taurine levels among mongoloid and non-mongoloid retardates studied 3 weeks apart

	Subject no.	Mongoloids 1	2	3	4	Means	Non-mongoloids 1	2	3	4	Means
		µmoles/liter					*µmoles/liter*				
Collection	1	310	199	203	215	232	116	151	65	127	115
Collection	2	150	169	211	134	166	96	128	100	93	104
Means		230	184	207	174	199	106	140	84	110	110

TABLE 7

Fasting serum values among mongoloid and non-mongoloid retardates matched for age, sex and weight

Collection day		Mongoloid male	Control male	Mongoloid female	Control female
		log_{10} taurine in µmoles/liter			
1	N	9	9	11	11
	$\bar{X}$	2.19	2.06	2.26	2.22
	Var.	0.037	0.016	0.015	0.019
2	N	14	12 [1]	9	9
	$\bar{X}$	1.98	1.94	2.09	2.04
	Var.	0.016	0.018	0.006	0.003

[1] Two non-Caucasians inadvertently matched were excluded.

same day. Subjects were fasted overnight, and blood specimens were drawn one hour after rising. Precision of the determination is within 10 µmoles/liter. The means based on log conversions of the serum values in micromoles per liter are presented in table 7. Bartlett's test for homogeneity of variances was applied prior to an overall analysis. The results indicated a highly significant heterogeneity, stemming primarily from the relatively large variance of the mongoloid males on day 1 and the small variances of mongoloid and control females on day 2. Though the obvious heterogeneity of variances discouraged pooling of data, an overall analysis supported the impression that the differences due to days exceeded any other source of variability. One can note suggestions of sex differences and of differences between mongoloid and controls. The ranking of means for all 4 groups agree for both days. If there are differences in mean serum taurine levels between mongoloid and control subjects and between sexes, it appears that the differences must be small. In several other studies in which serum taurine values have been obtained, the mongoloid means for both sexes have consistently been slightly higher than corresponding control means, but the differences have not reached conventional significance levels. Larger differences between days have precluded pooling these experiments.

When examining the raw data, one is impressed with a clustering of values within a given group. To test this possibility, logs of values were converted to relative deviates by taking the difference between each variable and the mean for its group and dividing by the standard deviation for that group. Figure 4 shows a plot of these data for mongoloid males (days 1 and 2 combined). Clearly, there is a suggestion of bimodality, but again, when the total data were analyzed, the obvious lack of values in the center of the distribution and the excess at the extremes does not reach statistical significance ($\chi^2 = 10.3$, $P > 0.25$). The suggested bimodality may reflect the bimodality already demonstrated in urinary taurine excretion by mongoloids (21). This heterogeneity could also account for the wide differences in variances observed in these subsamples and

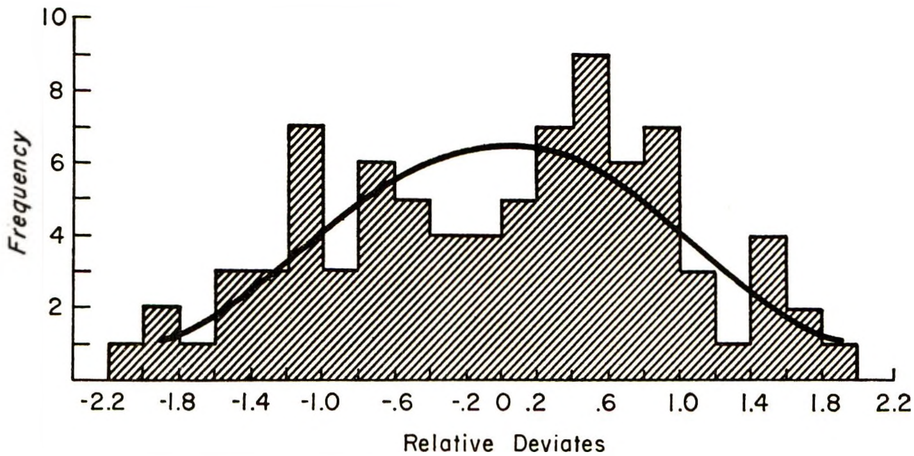

Fig. 4 Relative deviates of serum taurine values among pooled mongoloid and non-mongoloid retardates (N = 84); histogram is observed values, curved line, theoretical expectancies.

render statistical tests based on unimodal distributions meaningless. Hopefully, appropriate loading studies of the type illustrated in table 3 may distinguish subpopulations of serum or urinary taurine levels.

Although these findings on serum taurine levels appear inconclusive, they are not unexpected if the observed low urinary excretion of taurine by many mongoloids is a function of the kidney. Taurine is absorbed by these mongoloids (26), taurine tolerance curves after 4 or 40 mg taurine/kg body weight were normal, and it is certain that they ingest dietary taurine. Nevertheless, despite the lower mean urinary excretion of taurine by mongoloid subjects, the taurine level of the serum may be readily maintained within the wide range observed in sera by a diminution in the already slight production of metabolic taurine from methionine and cysteine or by increased conversion of taurine to its catabolites, isethionic acid and sulfate, both of which are rapidly excreted by the kidney. Further, the increased amount of sulfate possibly produced from taurine would be undetectable because of the large total excretion of sulfate.

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Effect of Vitamin B_6 Depletion on the Pyridoxal, Pyridoxamine and Pyridoxine Content of the Blood and Urine of Men [1]

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ABSTRACT The amount of pyridoxal, pyridoxamine and pyridoxine in the blood and urine of men was determined both when they were adequately nourished with vitamin B_6 and when they were depleted of the vitamin. When subjects were given 1.66 mg vitamin B_6 daily, pyridoxal made up about 60% and pyridoxamine, 35% of the urinary vitamin B_6. When the subjects were depleted of vitamin B_6, pyridoxal and pyridoxamine were found in the urine in approximately equal amounts. Little or no pyridoxine was detected in the urine when the daily vitamin B_6 intake was 1.66 mg or less. The amount of protein intake did not affect the form in which vitamin B_6 was excreted. Pyridoxal was the only form of vitamin B_6 found in the blood.

Little information is available concerning the distribution of pyridoxal, pyridoxamine, and pyridoxine in the urine and blood of normal humans. This has been due, in part at least, to lack of methods sufficiently sensitive for determining the individual forms of vitamin B_6. The microbiological differential assay procedure of Rabinowitz and Snell (1), when applied to human urine, resulted in negative values for pyridoxine and in low recoveries for pyridoxine added to urine. Fujita and Fujino (2) proposed a fluorometric determination of the forms of vitamin B_6 as the 4-pyridoxic acid lactone following chromatographic separation and oxidation. This method has been criticized because the procedure is not easy to duplicate and conversion of the different forms of the vitamin to the 4-pyridoxic acid lactone is not always complete. Toepfer and Lehmann (3) described a method for assay of vitamin B_6 in foods in which chromatographic separation of the different forms of vitamin B_6 was followed by microbiological assay with *Saccharomyces carlsbergensis*. This method was utilized in the present study to detemine the amount of pyridoxal, pyridoxamine and pyridoxine in the blood and urine of men both when they were adequately nourished with vitamin B_6 and when they were depleted of the vitamin. The 4-pyridoxic acid content of the urine was also determined.

EXPERIMENTAL

Procedure. In two separate human metabolic studies, men were fed a diet low in vitamin B_6 which contained either 54 or 150 g of protein daily. The daily diet fed to the subjects of study 1 contained 0.16 mg vitamin B_6, 54 g protein and 0.83 g of added methionine; vitamin-free casein, gelatin and the ordinary foods of the diet contributed, respectively, 5.0, 2.5 and 1.5 g nitrogen daily. The diet fed to the subjects of study 2 contained 0.16 mg vitamin B_6, 150 g of protein and 2.5 g added methionine; of the 24 g of nitrogen in the diet, casein supplied 15.0, gelatin 7.5 and ordinary foods 1.5 g. Both diets contained a mixture of animal and vegetable fats fed in such amounts that 40% of the caloric intake of each subject came from fat. More complete details concerning the composition of the diets and the methods of preparation have been given (4, 5).

Five men who ranged in age from 20 to 25 years, in height from 168 to 191 cm and in weight from 64 to 73 kg were the subjects of study 1; the 6 subjects of study 2 ranged in age from 18 to 31 years,

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in height from 173 to 188 cm and in weight from 63 to 71 kg. A description of the individual subjects has been given (5).

Subjects of study 1 consumed the experimental diet supplemented daily with 1.5 mg of pyridoxine for the first 6 days of the study, then the diet without vitamin B_6 supplementation for 40 days and, finally, the diet supplemented with 0.6 mg of of pyridoxine for 7 days. For the first 18 days the subjects of study 2 were given supplements of 1.5 mg of pyridoxine daily; then the supplement was withdrawn and the subjects were depleted of vitamin B_6 for 17 days, and then for 16 days they were given supplements of 0.6 mg of pyridoxine daily. Finally on each of two consecutive days the subjects of study 2 were given 50 mg of pyridoxine.

Methods. For vitamin B_6 assays urine samples were autoclaved in 0.055 N HCl for 3 hours and whole blood samples were autoclaved in 0.1 N HCl for 5 hours. The urine and blood samples of each individual were analyzed separately. Total vitamin B_6 content of the acid hydrolysates as determined using *S. carlsbergensis* (ATCC 9080) as the test organism. Aliquots of the hydrolysates were applied to columns of Dowex 50W-X8 in the potassium form and pyridoxal, pyridoxine and pyridoxamine were eluted with potassium acetate buffers. The procedure was as described by Toepfer and Lehmann (3). The amounts of pyridoxal, pyridoxamine and pyridoxine in the eluates were then determined using *S. carlsbergensis* (3). The 4-pyridoxic acid content of the urine from the subjects of study 1 was determined using the method of Reddy et al. (6); for study 2 the modification by Woodring et al. (7) of the Reddy method was used.

RESULTS

The average amounts of total vitamin B_6 and of pyridoxal, pyridoxamine and pyridoxine excreted by subjects fed the 54-g protein diet and by those fed the 150-g protein diet are shown in figures 1 and 2, respectively. The values for 4-pyridoxic acid are given in table 1. The values for total vitamin B_6 obtained from the sum of the chromatographed values for pyridoxal, pyridoxamine and pyridoxine agreed well with those obtained from urine samples not put through the chromatographic columns.

Before the experiments began the subjects of study 1 and of study 2, while ingesting self-selected diets, excreted 116 and 120 μg of vitamin B_6, respectively, per 24 hours. When supplements of 1.5 mg of pyridoxine were given daily for 6 days to the subjects consuming the experimental diet low in protein, the urinary vitamin B_6 values were essentially the same as those found when the subjects consumed self-chosen diets. Subjects fed the experimental diet high in protein supplemented with 1.5 mg of pyridoxine for 18 days excreted significantly more vitamin B_6 than they did while ingesting self-chosen diets ($P < 0.005$).

Pyridoxal was the form of the vitamin excreted in the largest amount when the subjects were consuming the experimental diets supplemented with 1.5 mg of pyridoxine or when they were ingesting self-selected diets; at these times pyridoxal

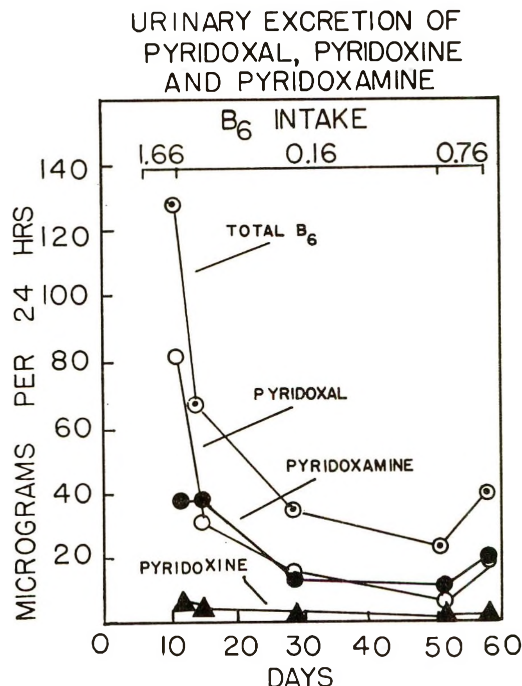

Fig. 1 Effect of the level of intake of vitamin B_6 on the urinary excretion of pyridoxal, pyridoxamine and pyridoxine by subjects fed the 54-g protein diet.

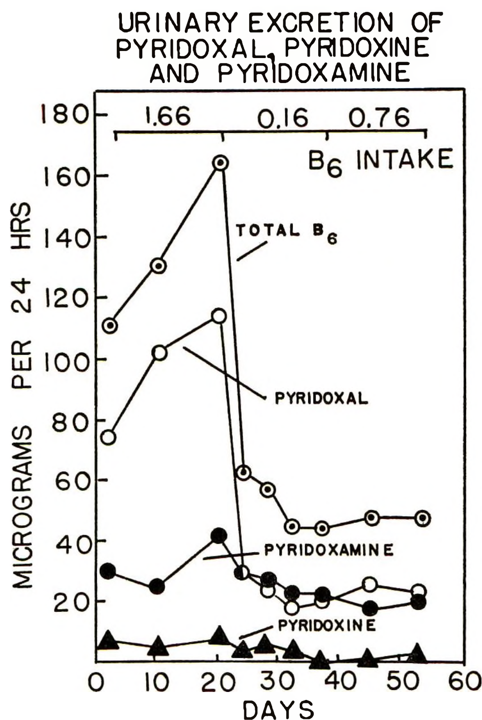

Fig. 2 Effect of the level of intake of vitamin B_6 on the urinary excretion of pyridoxal, pyridoxamine and pyridoxine by subjects fed the 150-g protein diet.

TABLE 1

The 4-pyridoxic acid excretion as affected by level of vitamin B_6 intake and protein intake

No. of days on specified vitamin B_6 intake	Vitamin B_6 intake	4-Pyridoxic acid excretion
	mg/day	*mg/day*
	Low protein diet	
6	1.66	1.01 ± 0.32 [1]
2	0.16	0.67 ± 0.30
5	0.16	0.18 ± 0.18
20	0.16	0.11 ± 0.08
40	0.16	0.05 ± 0.08
7	0.76	0.12 ± 0.05
	High protein diet	
8	1.66	0.88 ± 0.11
2	0.16	0.46 ± 0.04
5	0.16	0.25 ± 0.13
17	0.16	0.25 ± 0.02
13	0.76	0.25 ± 0.02
2	50.16	26.20 ± 3.42

[1] SD.

accounted for approximately 65% and pyridoxamine, for 30% of the urinary vitamin, with only negligible amounts of pyridoxine being noted. Urinary 4-pyridoxic acid accounted for approximately 50% of the 1.66 mg intake of vitamin B_6.

When the diets containing 0.16 mg vitamin B_6 were introduced to the subjects, the amount of the vitamin and of 4-pyridoxic acid excreted by them decreased rapidly; and after 4 to 5 days of vitamin B_6 deprivation the subjects excreted approximately 40% as much vitamin B_6 and 20% as much 4-pyridoxic acid as they did when given the diets containing 1.66 mg of the vitamin. These decreases were highly significant; $P < 0.01$ for vitamin B_6 values and $P < 0.005$ for 4-pyridoxic acid values. The amount of pyridoxal in the urine decreased at a much faster rate than that of pyridoxamine; the subjects deprived of vitamin B_6 for 4 days excreted approximately equal amounts of pyridoxal and pyridoxamine. The amounts of these 2 substances continued to be excreted in equal amounts as the period of vitamin B_6 deficiency progressed.

During the repletion periods supplements of 0.6 mg of pyridoxine caused a slight increase in the amounts of pyridoxal and pyridoxamine excreted by subjects fed the low protein diet but had no effect on the amounts excreted by those given the high protein diet; pyridoxal and pyridoxamine were excreted in equal amounts by both groups of subjects. At the end of the study when subjects fed the high protein diet were given supplements of 50 mg of pyridoxine on each of two consecutive days, on the second day they excreted 4% of the dose as pyridoxal, 10% as pyridoxine, 2% as pyridoxamine and 50% as 4-pyridoxic acid.

Table 2 shows that an intake of 0.16 mg of vitamin B_6 daily caused a rapid decrease in the vitamin B_6 content of the blood of the subjects of both studies. Acid hydrolysates of whole blood of the subjects given 150 g of protein daily were applied to chromatographic columns for separation of the different forms of vitamin B_6. The blood of the subjects fed 54 g of protein was not chromatographed, and the values

TABLE 2

Vitamin B_6 content of blood as affected by level of vitamin B_6 intake and protein intake

No. of days on specified vitamin B_6 intake	Vitamin B_6 intake	Vitamin B_6 in blood
	mg/day	*µg/100 ml*
	Low protein diet [1]	
6	1.66	0.72 ± 0.28 [2]
10	0.16	0.31 ± 0.10
20	0.16	0.24 ± 0.05
39	0.16	0.14 ± 0.02
6	0.76	0.34 ± 0.17
	High protein diet [3]	
18	1.66	0.66 ± 0.08
8	0.16	0.22 ± 0.05
16	0.16	0.20 ± 0.05
15	0.76	0.26 ± 0.03

[1] Values given for vitamin B_6 are for total vitamin B_6 and were obtained from unchromatographed samples.
[2] SD.
[3] Values given for vitamin B_6 are those obtained for pyridoxal, the only form of vitamin B_6 found in the blood.

given represent the total vitamin B_6 content. Only pyridoxal was found in the blood when the subjects were given daily 1.66 mg or less of vitamin B_6; however, blood samples from fasting subjects who had been given 50 mg of pyridoxine on each of two consecutive days contained from 4.0 to 5.5 µg of pyridoxal and from 0.5 to 0.8 µg of pyridoxine per 100 ml of blood. No pyridoxamine was found.

DISCUSSION

When the vitamin B_6 intake of the subjects of the present studies approximated the amount normally consumed by man, pyridoxal made up about two-thirds and pyridoxamine one-third of the vitamin B_6 excreted in the urine. But, when the dietary intake of vitamin B_6 was severely restricted, the subjects excreted approximately equal amounts of pyridoxal and pyridoxamine.

When the intake of vitamin B_6 was about 1.6 mg daily, the form in which the vitamin was ingested did not affect the form in which it was excreted. Subjects of the present studies excreted approximately twice as much pyridoxal as pyridoxamine both when they were fed the experimental diets supplemented with 1.5 mg of pyridoxine and when they were consuming self-selected diets that contained about the same amount of vitamin B_6. In the latter instance the forms of vitamin B_6 ingested were probably pyridoxal and pyridoxamine (1, 8).

In contrast, when large amounts of vitamin B_6 were consumed, the form fed determined to some extent the form which was excreted. In the present study urinary pyridoxal and pyridoxine accounted for 4% and 10%, respectively, of a 50-mg dose of pyridoxine. Similar results were obtained by Rabinowitz and Snell (9) following loading of human subjects with 82 mg of pyridoxine. The latter authors showed that feeding 70 mg of pyridoxamine caused significant increases in urinary pyridoxal and pyridoxamine and that feeding 82 mg of pyridoxal caused increases mainly in pyridoxal.

That the vitamin B_6 content of the blood and urine of subjects fed the low protein diet increased slightly when they were given 0.6 mg of vitamin B_6 daily following the period of vitamin depletion, whereas that of subjects fed the high-protein diet did not, indicates the vitamin B_6 requirement of man is affected by the level of protein in the diet. However, the concentration of vitamin B_6 in the blood and urine decreased so rapidly when dietary vitamin B_6 was restricted that it was difficult to determine whether the level of protein intake affected the rate of decrease.

After 18 days of supplementation with 1.5 mg of vitamin B_6 subjects fed the high protein diet excreted larger amounts of pyridoxal and pyridoxamine than they did while consuming self-selected diets. The progressive elevation of urinary vitamin B_6 when the intake was 1.66 mg per day might indicate that this level of intake was higher than that normally consumed by the subjects. Feeding this amount of vitamin B_6 for 6 days to subjects given the low protein diet did not, however, cause an increase in urinary vitamin B_6.

That pyridoxamine was excreted in the urine of the subjects but was not present in the blood indicates that transamination takes place in the kidney. Transaminase activity has been reported to be present in the kidney of rats as well as in liver and other tissues (10, 11).

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Growth and Concentrations of Amino Acids in Plasma of Rats Fed Four Levels of Amino Acids [1,2]

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ABSTRACT Amino acids in plasma and liver were measured to determine whether they would reflect dietary intakes of weanling rats fed 4 levels of amino acids in the same proportions when blood was sampled at an interval of fasting at which it was least affected by either absorption or starvation. To determine the interval of fasting to be used, animals fed casein were fasted 3, 6, 9 or 12 hours in the first experiment. Plasma obtained after the 6-hour interval appeared to be least affected by absorption or starvation since it contained neither the highest nor lowest levels of most ninhydrin-reacting materials and contained few materials formed during metabolism. In the second experiment diets were formulated to contain 115, 100, 85 or 70% of proposed minimal requirements. Although the nitrogen intake of rats fed the lowest level of amino acids was only 56% of the highest level, only the concentration of lysine in plasma was related directly to intake at all levels, and that of threonine was related at the three lower levels. Plasma concentrations of other amino acids and ninhydrin-reacting materials are discussed in relation to intake and to levels in the liver. Carcass nitrogen was the only parameter of growth that was related directly to intake of all levels of amino acids.

The significance of the concentrations of free amino acids in the nonprotein fraction of the blood has received considerable attention during the last decade. The quantitative relationships between the composition of ingested protein and the plasma amino acids have been of particular interest because a method of predicting the limiting amino acid or acids in foods from concentrations in plasma has been sought.

Although many investigators have reported plasma amino acid levels for rats, the data are difficult to interpret because of interlaboratory differences in experimental conditions. Among the many factors that affect concentrations of amino acids in plasma are: food intake, digestion and absorption, fasting, previous protein level, amino acid deficiencies or excesses, and supplementary amino acids. Age, species and source of blood also have an influence. For the most part, observations that a high level of an amino acid in the plasma was associated with a dietary excess of that amino acid have been made when only one or a few dietary amino acids were increased rather than when all amino acids were increased simultaneously. Also, plasma amino acids have frequently been determined for non-fasted animals in which digestion or absorption may have affected the results or for animals fasted for 12 or 18 hours when starvation may have influenced the levels. No data concerning plasma amino acids or growth have been found for animals fed all amino acids in the same proportions but at different levels. In addition, plasma levels have seldom been examined in relation to parameters of growth.

In the present experiment the effect on the concentrations of plasma amino acids and related metabolites of consuming regularly increasing intakes of amino acids in proportions that would promote satisfactory growth in young rats was investigated and at the same time growth and deposition of nitrogen were measured. The minimal requirements for essential

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amino acids that were reported initially by Rama Rao and co-workers (1–3) and later modified by Ranhotra and Johnson (4) were accepted as representing a balanced mixture of essential amino acids that could be fed at various levels. Data obtained in this initial experiment then could be used as a basis for further exploration of the effect of variable concentrations of individual amino acids.

EXPERIMENTAL

Experiment 1 was designed to determine whether concentrations of plasma amino acids varied significantly between different intervals of fasting, and to choose the time after food was removed at which plasma amino acid levels appeared to be least affected by either absorption or starvation. It is to be expected that plasma obtained shortly after the ingestion of food would reflect the rapid influx of amino acids into the bloodstream, whereas blood withdrawn after a prolonged period of fasting would contain principally amino acids withdrawn from metabolic pools. Plasma obtained at either of these intervals would be less useful in evaluating the nutritive state of the animal than plasma sampled at an intermediate time when the concentrations of amino acids and related metabolites were not influenced unduly by either recent ingestion or prolonged absence of dietary amino acids. It is postulated that the nutritive quality of a protein or mixture of amino acids should be reflected most accurately at such an intermediate interval.

Experiment 2 was designed to determine whether the concentrations of amino acids in plasma would reflect the dietary intakes of amino acids when 4 levels of essential and nonessential amino acids were administered to rats. Proportions of amino acids in the diets were kept constant as total levels were altered to avoid the effect of competition during absorption or the disturbance of metabolic processes that might follow a decrease in single amino acids. Young animals were used because their higher tissue requirements for amino acids might cause the concentrations of amino acids to be more sensitive to dietary level than those of adults animals. Food consumption, weight gain, carcass nitrogen, liver nitrogen and liver lipid were measured, as well as the concentrations of ninhydrin-reacting materials in plasma and liver.

Diets. In experiment 1, designed to study the effect of different intervals of fasting, 13.6% of vitamin-free casein which was analyzed for amino acids by ion-exchange chromatography was incorporated in the diet. This amount of casein equalled in weight the amino acid mixture fed at the 100% level in the second experiment (table 1).

In experiment 2, the amounts of essential amino acids proposed as minimal requirements by Rama Rao and co-workers (1–3) and Ranhotra and Johnson (4) were selected for modification because they were reported to induce good growth. Mixtures of amino acids that furnished 115, 100, 85 and 70% of these quantities of essential amino acids were prepared to determine the effect on plasma concentrations of administering amounts of amino acids that were above as well as below the minimal requirements but in the same proportions. Since a preliminary test indicated that similar results in respect to growth and plasma amino acids were obtained whether the nonessential amino acids were patterned after casein or after data obtained from examination of 15 representative dietary proteins, the nonessential amino acids were patterned after casein as suggested by Rama Rao et al. (5), except that part of the proline was replaced by glutamic acid. Fat was limited to 2% when amino acids were fed because preliminary data indicated that food consumption was reduced by 6% when the fat level was 10%. The composition of all diets is given in table 1.

Animals. Male weanling rats of the Sprague-Dawley strain were housed individually, with food and water being supplied ad libitum. In experiment 1, 24 animals initially weighing 47 g were fed the casein diet for 12 days. In experiment 2, 6 animals weighing 53 g were assigned to each of the 4 amino acid diets and fed for 21 days. Food consumption and weight gain were averaged for each week and for the entire experimental period. Food consumption, weight gain and total carcass nitrogen were calculated for indivi-

TABLE 1
Composition of diets

	Dietary level of amino acids				Casein
	115%	100%	85%	70%	
L-Arginine·HCl	0.55	0.48	0.41	0.34	0.47
L-Cystine	0.39	0.34	0.29	0.24	0.04
L-Histidine·HCl·H_2O	0.36	0.31	0.26	0.21	0.37
L-Isoleucine	0.63	0.55	0.47	0.39	0.70
L-Leucine	0.81	0.70	0.59	0.48	1.23
L-Lysine·HCl	1.30	1.13	0.96	0.79	1.05
L-Methionine	0.18	0.16	0.14	0.12	0.36
L-Phenylalanine	0.48	0.42	0.36	0.30	0.66
L-Threonine	0.58	0.50	0.42	0.34	0.53
L-Tryptophan	0.17	0.15	0.13	0.11	—
L-Tyrosine	0.35	0.30	0.25	0.20	0.73
L-Valine	0.63	0.55	0.47	0.39	0.77
DL-Alanine	0.54	0.47	0.40	0.33	0.37
L-Aspartic acid	1.17	1.02	0.87	0.72	0.92
L-Glutamic acid	4.63	4.03	3.43	2.83	3.05
Glycine	0.35	0.30	0.25	0.20	0.26
L-Proline	1.38	1.20	1.02	0.84	1.33
DL-Serine	1.13	0.98	0.83	0.68	0.75
(Total amino acids)	(15.63)	(13.59)	(11.55)	(9.51)	(13.59)
Vitamin-dextrin mixture [1]	15.00	15.00	15.00	15.00	15.00
Salts 446 [2]	4.00	4.00	4.00	4.00	4.00
Cellulose	2.00	2.00	2.00	2.00	2.00
Cottonseed oil	2.00	2.00	2.00	2.00	10.00
Dextrin	61.37	63.41	65.45	67.49	55.41
Total	100.00	100.00	100.00	100.00	100.00
Nitrogen					
Essential amino acids	0.87	0.75	0.64	0.53	
Nonessential amino acids	1.03	0.90	0.76	0.63	
Total	1.90	1.65	1.40	1.16	2.16

[1] Modified from Rama Rao et al. (2). Each 15 g of vitamin-dextrin mixture contained the following in milligrams: α-tocopheryl acetate, 10.9; ascorbic acid, 1.5; biotin, 0.015; 0.1% trituration of crystalline vitamin B_{12} in mannitol, 0.5; Ca pantothenate, 7.5; choline chloride, 99.0; folic acid, 0.3; inositol, 49.5; menadione, 0.015; niacin, 3.0; *p*-aminobenzoic acid, 7.5; pyridoxine·HCl, 0.75; riboflavin, 1.5; thiamine·HCl, 1.5; vitamins A and D_2 (500,000 IU A acetate and 50,000 IU D_2/g), 0.8; and dextrin to make a total of 15 g.

[2] Spector, H., J. Biol. Chem., 173: 659, 1948.

dual animals but 3 animals were treated as a single sample for determination of plasma amino acids and of nitrogen, lipid and amino acids in liver.

Fasting. In experiment 1, which was related to fasting, food cups were removed either 3, 6, 9 or 12 hours before blood samples were drawn. On the basis of data obtained in this experiment, food was removed from all animals 6 hours before blood was drawn in experiment 2.

Analyses. After fasting, animals were anesthetized by intraperitoneal injection of 0.125 ml of sodium pentobarbital (15 mg/ml) per 100 g of body weight and then decapitated. Blood drained from the carotid artery and jugular veins was pooled and placed in an ice bath. Livers were then removed immediately and representative samples from 3 animals were pooled for determination of amino acids, nitrogen, lipid and moisture.

Plasma was prepared for analysis by the methods of Hamilton and Van Slyke (6) and Stein and Moore (7) except that some modifications suggested by Chance [5] were incorporated. Liver tissues were prepared for analysis by the methods of Hamilton (8) and Tallen et al. (9). Concentrations of ninhydrin-reacting materials in samples that represented approximately 0.5 ml of plasma or 0.01 g of wet tissue

[5] Chance, R. E. Doctoral thesis, 1962. Purdue University, Lafayette, Indiana.

were determined with a Beckman Model 120B automatic amino acid analyzer equipped with a high sensitivity cuvette. Nitrogen in diets and livers was determined by the boric acid modification of the macro-Kjeldahl method. Each carcass, minus blood and liver, was dried to constant weight and the nitrogen content was calculated from body water lost during drying, by the method of Miller and Bender (10). Liver lipid was determined by the method of Bligh and Dyer (11). Data were treated statistically by analysis of variance.

RESULTS AND DISCUSSION

Experiment 1

Effect of fasting on plasma amino acids. The concentrations of ninhydrin-reacting materials after 3, 6, 9 or 12 hours of fasting are shown in table 2. Differences were observed in the concentrations of most of the materials between all intervals of fasting. Significant variation at the 1% level was found for valine, glycine, proline, asparagine-glutamine and α-amino-*n*-butyric acid, and at the 5% level for histidine, isoleucine, leucine, threonine, tryptophan and alanine. Lysine and citrulline increased up to 6 hours and then decreased. β-Alanine, glutamic acid and urea increased steadily for 12 hours, whereas aspartic acid and proline decreased. Carnosine, creatinine, 1-methylhistidine and 3-methylhistidine appeared only after 9 or 12 hours of fasting. All others increased up to 9 hours and then decreased, except

TABLE 2

Concentration of ninhydrin-reacting materials in plasma after 3, 6, 9 and 12 hours of fasting [1]

Ninhydrin-reacting material	Hours fasted			
	3	6	9	12
	μmoles/ml			
Arginine	0.100	0.152	0.186	0.107
½ Cystine	0.014	0.014	0.006	0.012
Histidine	0.065	0.082	0.048	0.064
Isoleucine	0.070	0.075	0.090	0.066
Leucine	0.075	0.094	0.110	0.087
Lysine	0.484	0.585	0.534	0.472
Methionine	0.017	0.029	0.036	0.022
Phenylalanine	0.038	0.048	0.053	0.046
Threonine	0.772	0.888	0.986	0.805
Tryptophan	0.028	0.038	0.042	0.020
Tyrosine	0.052	0.068	0.082	0.046
Valine	0.150	0.151	0.165	0.129
Alanine	0.476	0.477	0.502	0.278
Aspartic acid	0.015	0.014	0.013	0.010
Glutamic acid	0.156	0.164	0.191	0.217
Glycine	0.378	0.395	0.493	0.391
Hydroxyproline	0.040	0.055	0.063	0.028
Proline	0.236	0.211	0.200	0.139
Serine	0.470	0.537	0.561	0.461
Asparagine-glutamine	0.744	0.775	1.235	0.484
β-Alanine	0.009	0.014	0.018	0.027
α-Amino-*n*-butyric acid	0.012	0.017	0.052	0.025
Ammonia	0.359	0.382	0.440	0.426
Carnosine	tr	tr	tr	0.009
Citrulline	0.134	0.174	0.114	0.084
Creatinine	tr	tr	tr	0.230
Ethanolamine	0.032	0.055	0.045	0.063
1-Methylhistidine	—	—	0.005	0.004
3-Methylhistidine	—	—	0.009	0.007
Ornithine	0.064	0.070	0.082	0.063
Phosphoethanolamine	0.009	0.011	0.010	0.016
Phosphoserine	0.018	0.014	0.024	0.015
Taurine	0.046	0.051	0.063	0.062
Urea	3.180	3.670	4.000	4.320

[1] Values represent the average of 2 pooled samples; 3 animals, average weight 97 g, pooled for each sample; casein diet, 12-day feeding period.

cystine, histidine and phosphoserine which did not follow a consistent pattern.

Maximal values for plasma amino acids also were observed between 9 and 12 hours of fasting by Henderson et al. (12) who followed 13 materials in plasma of 200- to 300-g rats during a 24-hour period. Peak levels of arginine, phenylalanine and tryptophan occurred after the same interval of fasting as in the present study. Differences in plasma amino acid levels of human beings and animals at various intervals after the ingestion of food have been reported by several workers (13–22).

Increasing amounts of materials formed during metabolism after 9 and 12 hours of fasting (table 2) suggested that catabolism was taking place and that concentrations of amino acids in blood sampled at either of these intervals probably would be affected by starvation rather than by quantities of amino acids administered previously. Samples from the 3-hour fasting interval contained either the highest or lowest amounts of 16 materials, indicating that concentrations might have been affected by recent food intake. It was recognized that amino acids might be absorbed at different rates from casein and from the mixtures of amino acids tested in the second experiment. Nevertheless the concentrations of individual amino acids and metabolites would change gradually and the trends should be in the same direction whether an intact protein or a mixture of crystalline amino acids was consumed.

The 6-hour fasting interval appeared to be the time after ingestion of food at which concentrations of almost all compounds were least affected by either absorption or starvation since neither highest nor lowest levels were observed. This interval was judged to be most suitable for the second experiment when the effects on plasma amino acids of different concentrations of dietary amino acids were compared. A 6-hour fast also was used by McLaughlan and Illman (23) for estimation of amino acid requirements of the growing rat.

Experiment 2

Effect of 4 levels of amino acids on plasma amino acids. The quantities of amino acids incorporated in the diets (table 1) represented 115, 100, 85 and 70% of those reported as minimal requirements by Rama Rao and associates (1–4). Mean daily food intakes of the rats fed these diets were 11.9, 11.6, 11.6 and 10.9 g, respectively. The average amounts of amino acids consumed by the rats are presented in table 3. Rats offered 70% of the minimal requirements consumed 56% as much of each amino acid as those fed the highest level of amino acids instead of 61% as planned.

The concentrations of amino acids in plasma are also shown in table 3. Of the essential amino acids, only lysine was related directly to intake at all 4 levels, the differences being highly significant ($P < 0.01$). The concentration of threonine was also related directly to intake at the three lower levels. Leucine and valine were unchanged. Although other essential amino acids tended to decrease as intakes were lowered, changes were small in comparison with the 44% reduction in intakes.

No consistent relationships between intakes and plasma levels of nonessential amino acids were evident. Of the other ninhydrin-reacting compounds, the concentration of ethanolamine appeared to be related directly to intake, whereas the concentrations of citrulline and taurine were related inversely except in the animals fed the highest level of amino acids. The differences for citrulline were statistically significant ($P < 0.05$).

High plasma levels of several amino acids as a result of high dietary intakes as well as low levels associated with low intakes have been reported in different species. In the present experiment, lysine was the only amino acid that confirmed the reports that an increased intake of an amino acid is followed by a high plasma level; and only lysine and threonine confirmed the observations that an amino acid deficit results in low plasma levels. That the results with only these two amino acids agreed with those of other workers may be attributed in part to the latter being based on plasma from nonfasted animals. Results obtained in experiment 1 (table 2) and reports by other investigators (12–22) emphasize the effect of absorption of food or of starvation on

TABLE 3

Average daily intakes and concentrations of amino acids in plasma and liver of rats fed 4 levels of amino acids in the same proportions [1]

	115% level of AA			100% level of AA			85% level of AA			70% level of AA		
	Intake	Plasma	Liver	Intake	Plasma	Liver	Intake	Plasma	Liver	Intake	Plasma	Liver
	µmoles	*µmoles/ml*	*µmoles/g*	*µmoles*	*µmoles/ml*	*µmoles/g*	*µmoles*	*µmoles/ml*	*µmoles/g*	*µmoles*	*µmoles/ml*	*µmoles/g*
Arginine	314	0.088		266	0.096	11	226	0.105	tr	175	0.095	1
Cystine	221	0.016		188	0.020		160	0.013		125	0.013	tr
Histidine	222	0.047	13	186	0.045	16	157	0.046	16	126	0.038	15
Isoleucine	571	0.046	5	486	0.048	15	415	0.048	18	324	0.052	13
Leucine	735	0.074	10	619	0.076	33	521	0.073	40	399	0.070	30
Lysine	813	0.458	23	691	0.408	45	585	0.365	42	455	0.284	35
Methionine	143	0.021	2	125	0.017	10	108	0.016	14	88	0.016	8
Phenylalanine	346	0.046	4	295	0.044	14	252	0.039	18	198	0.036	14
Threonine	579	0.508	28	486	0.509	45	409	0.424	46	312	0.342	37
Tryptophan	99	0.037		85	0.046		74	0.028		58	0.022	
Tyrosine	230	0.057	4	192	0.046	13	160	0.056	17	120	0.049	14
Valine	640	0.123	10	545	0.122	28	465	0.122	34	363	0.122	26
Alanine	722	0.454	102	612	0.403	120	521	0.494	130	404	0.446	118
Aspartic acid	1046	0.012	20	889	0.015	33	758	0.014	35	590	0.015	25
Glutamic acid	3771	0.125	193	3200	0.090	173	2723	0.121	171	2112	0.114	157
Glycine	554	0.207	50	463	0.249	89	386	0.234	68	290	0.259	56
Hydroxyproline		0.043			0.048			0.041			0.033	
Proline	1426	0.155	8	1209	0.140	25	1028	0.153	32	796	0.141	23
Serine	1280	0.486	51	1082	0.480	73	916	0.540	86	705	0.490	77
Asparagine-glutamine		0.910	59		0.902	84		0.998	110		0.974	105
α-Amino-*n*-butyric acid		0.013	2		0.014	3		0.015	3		0.010	2
Ammonia		0.224			0.201			0.237			0.208	
β-Alanine			13			15			10			7
β-Aminoisobutyric acid			1			2			1			1
Citrulline		0.077			0.087			0.089			0.114	
Ethanolamine		0.026	75		0.022	68		0.022	32		0.020	32
γ-Aminobutyric acid			2			3			4			3
Glycerophosphoethanolamine			4			6			4			3
Ornithine		0.055	10		0.061	28		0.058	30		0.065	26
Phosphoethanolamine		0.016	9		0.015	20		0.022	9		0.021	12
Phosphoserine		0.010	32		0.008	22		0.009	25		0.008	25
Taurine		0.171	52		0.172	16		0.189	10		0.200	12
Urea		1.605	41		2.235	67		2.210	65		1.635	54

[1] Intakes are the average in micromoles for 6 animals for 21 days. Plasma in micromoles per milliliter and liver in micromoles per gram of wet tissue are the average of 2 samples of 3 animals each.

plasma amino acid levels. However, when this effect was minimized in the present study by sampling the blood after 6 hours of fasting, concentrations of most ninhydrin-reacting materials varied little in response to differences in dietary levels of amino acids. Furthermore, the feeding of large excesses or severely limited amounts of single amino acids, as reported by some investigators, may have resulted in unbalanced amino acid mixtures; hence the findings in respect to concentrations of plasma acids would bear little relationship to levels observed when the intakes of all amino acids were increased or decreased simultaneously as in the present experiment.

Other factors affecting plasma amino acids may also have been responsible for the difference in findings. That age affects plasma amino acid levels has been observed in young and adult rats (17), in children (24), and also in young and elderly adults (25). Differences resulting from previous protein level (26–30) and from source of blood (13, 32, 33) have been reported.

Effects of 4 levels of amino acids on growth. Food intake, weight gain and body composition data are shown in table 4. Animals fed the 115, 100, or 85% diets consumed similar amounts of food, but those fed the 70% diet consumed one gram less per day than animals fed the 115% diet. Mean daily gains during the 21-day feeding period were 4.5, 3.7, 3.6 and 3.0 g, respectively. Differences in weight gain were highly significant ($P < 0.01$). The average final weight of animals fed the 115% diet was 147 g in comparison with 129 g for animals fed either the 100 or 85% diets and 117 g for animals fed the 70% level.

If the data pertaining to rats consuming the highest level of amino acids (115%) are expressed as 100%, nitrogen intakes of rats fed the mixtures designated as 100, 85 and 70% levels were 85, 72 and 56%, respectively, of the highest level; carcass nitrogen values were 93, 87 and 79%; and total liver nitrogen values were 87, 86 and 75%. In terms of gain in body weight and in carcass and liver nitrogen, the highest level of amino acids was the most satisfactory, thus indicating that growth of young rats can be improved by simultaneously feeding larger amounts of amino acids than those reported as minimal requirements (1–4). The decrease in intake to 85% of the minimal requirements caused little change in weight gain or liver nitrogen but reduced total carcass nitrogen. The reduction to 70% of the minimal values caused a decrease in all parameters, but the change was less severe than expected in view of the 44% decrease in dietary nitrogen. Liver lipid values were not influenced markedly by treatment.

Effect of 4 levels of amino acids on concentrations of amino acids in liver. Concentrations of ninhydrin-reacting materials in liver samples from animals fed the four amino acid diets appear in table 3. Glutamic acid and ethanolamine were the only compounds whose concentrations were related directly to intake, and none were related inversely. The lowest con-

TABLE 4

Mean food intakes, weight gains and body composition data for animals fed 4 levels of amino acids for 21 days [1]

	Dietary level of amino acids			
	115%	100%	85%	70%
Food consumption, g/day	11.9 ± 0.57	11.6 ± 0.56	11.6 ± 0.68	10.9 ± 0.62
Wt gain, g/day	4.5 ± 0.41	3.7 ± 0.25	3.6 ± 0.28	3.0 ± 0.18
Nitrogen intake, mg/day	228 ± 1.11	194 ± 0.94	165 ± 1.34	128 ± 0.72
Carcass nitrogen,[2] g	4.98 ± 0.20	4.61 ± 0.20	4.35 ± 0.30	3.93 ± 0.20
Gain in carcass nitrogen, g	3.63 ± 0.20	3.26 ± 0.20	3.00 ± 0.30	2.58 ± 0.20
Total liver nitrogen, mg	184 ± 10.1	160 ± 11.9	158 ± 10.3	138 ± 12.0
Gain in liver nitrogen, mg	132 ± 11.9	108 ± 10.1	106 ± 10.3	88 ± 12.0
Liver lipid, % dry wt	18.7 ± 0.34	18.4 ± 0.00	17.8 ± 3.30	19.1 ± 0.84

[1] Food consumption, weight gain, nitrogen intake and carcass nitrogen are the average of 6 animals; liver nitrogen and liver fat are based on 2 samples of 3 animals pooled; mean ± SD.
[2] Carcass minus blood and liver.

centrations of all essential and nonessential amino acids except glutamic acid were observed in livers of animals fed the 115% level of amino acids, whereas the highest concentrations of all amino acids except lysine and glycine were noted in animals fed the 85% level. Lysine and glycine were highest in livers of animals fed the 100% diet.

Concentrations of urea in both plasma and liver of animals fed 115% of the minimal levels of amino acids were low, being slightly lower than in animals fed the 70% diet. Since the animals fed the 115% level had the highest values for carcass and liver nitrogen, the concentrations of urea in plasma and liver were related inversely to nitrogen in the tissues. However, plasma urea values were lower in rats fed all mixtures of amino acids than in those fed casein (table 2) and also lower than in those reported in the literature for larger rats (34) or for animals fasted 12 hours (35).

The response of amino acids in plasma after a particular treatment appears to be influenced by age, species, time of taking blood after ingestion of food and the source of blood. When these factors were controlled, as in the present experiment, only lysine and threonine appeared to be affected markedly by dietary treatment. The fact that these are the only essential amino acids that do not participate extensively in transamination may be significant. However, the free amino acids of the plasma arise from absorption, synthesis and tissue breakdown which proceed continually. Consequently the amino acids in plasma represent a balance between rates of addition and removal or substitution. Therefore the metabolic and clinical significance of the free amino acids in the plasma should be considered only in relation to accompanying changes in tissue levels (36), and further investigation of concomitant changes in concentrations of amino acids in muscle, liver and plasma is necessary. Consideration should also be given to the effect on plasma and tissue levels and on all parameters of growth of administering well-balanced mixtures of amino acids and different levels of amino acids fed in the same proportions before a final statement concerning the usefulness of plasma amino acid data can be made.

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Effect of Glutamine on Inhibition of Rat Growth by Glycine and Serine [1]

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ABSTRACT Different requirements for glutamine of rats fed two amino acid diets were investigated in two rat-feeding experiments. In the first, the nonessential and essential amino acid mixtures were interchanged and the nonessential mixture was fed at 3 levels to determine whether the need for glutamine was related to the essential amino acids, the nonessential amino acids, or to their relative proportions. Results showed only the composition of the nonessential mixtures to be important. Because the mixtures differed primarily in their proportions of glycine and serine, these amino acids were tested in the second experiment by feeding 3 levels of each in all combinations, both with and without glutamine. Multiple regression analysis of the gains showed that additions of glycine or serine in excess of an undefined optimal level caused a linear reduction in gain and that the effects of each were additive. Inclusion of glutamine greatly reduced the inhibitory effects. Urine samples from selected groups were examined with an automatic amino acid analyzer. Increased excretion of a number of compounds resulted from feeding glycine and serine, which was prevented or greatly reduced by glutamine. These included, besides glycine and serine themselves, threonine, histidine, glutamine/asparagine, taurine, alanine, and lysine. The action of glutamine was accompanied by an increase in the excretion of urea and ammonia.

Previous publications from this laboratory demonstrated that relatively large amounts of arginine and glutamic acid were necessary for a high rate of gain of rats fed an amino acid diet (1, 2). It was shown that although either compound could spare the other to a large extent, the presence of both was required for highest gains. It was shown further that glutamine could be substituted for, and was more effective than glutamic acid in this diet.

In subsequent studies,[2] in which amino acids were fed in a different pattern from that used in the above experiments, it was noted that the requirement for glutamic acid or glutamine appeared to be greatly reduced.

The possibility that the requirement for glutamate or glutamine might be imposed by the particular balance of amino acids has been investigated. The results of two feeding studies reported here provide evidence that the need for glutamine in the diet depends upon the proportions of glycine and serine in the amino acid mixture.

EXPERIMENTAL

The same general procedures for the feeding studies were followed as described previously (1, 2). Weanling male rats of the Sprague-Dawley strain were fed amino acid diets over a 3-day adaptation period. Animals were divided into groups of 8 and assigned to experimental diets on the fourth day. Diets and distilled water were fed ad libitum for a 3-week period. Animals were housed individually in suspended wire-bottom cages and room temperature was maintained at 25°. Diets were weighed out daily and food consumption values corrected for spillage and moisture were determined for each week.

Diets. As in previous studies (1, 2), diets were formulated to contain 4% of salt mixture [3] and 5% of corn oil [4] with wheat starch [5] as the carbohydrate. Vitamins [6] were added in a portion of the starch and sufficient sodium bicarbonate

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[2] Unpublished data.

[3] Hegsted et al. 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459; obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

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

[5] Aytex, General Mills, Minneapolis.

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

was included to neutralize the hydrochlorides of arginine, histidine, and lysine. Basal diets were prepared with the above constituents together with an essential amino acid mixture to provide 50% of the diet. Experimental diets were prepared by adding the experimental variables under study and sufficient additional starch to total 100%. A gluten diet was fed as a control with each experiment. This consisted of 22.5% of gluten; 1% of L-lysine·HCl, 0.2% each of L-histidine·HCl·H_2O, DL-methionine, and DL-threonine; and 0.05% of DL-tryptophan in place of the amino acid mixtures of the experimental diets. Diets were moistened with distilled water at the rate of 250 ml/kg of dry diet during the final mixing. Spilled and unconsumed food portions were allowed to air-dry before weighing and food consumption was calculated to the air-dried basis.

Analysis of urine. Stainless steel metabolism cages were used for obtaining samples of urine from animals on selected diets. Each receiving flask contained 5 ml each of toluene and 0.2 M citrate buffer (pH 2.2). Samples were collected from 3 or 4 animals over periods of approximately 20 hours during the last week and were frozen until analyzed. Urine samples were pooled by group, adjusted to pH 2.2 and filtered through glass filter paper without treatment to remove ammonia or protein. Nitrogenous compounds were determined with a Beckman-Spinco Model 120-C amino acid analyzer following the normal procedure for physiological fluids.

Experiment 1. A requirement for glutamic acid or glutamine was demonstrated previously using basal diets A, B, C, and D (2). The amino acids of these basal diets were based originally on the proportions calculated to be present in wheat gluten, supplemented with essential amino acids (as in the gluten control diet), and with a portion of the proline replaced by glycine. They differed from one another principally in the isomeric form of certain amino acids as previously described (2). A much lower requirement for glutamic acid was found in subsequent work[7] using a diet (basal diet E) in which the essential amino acids were provided in proportion to the requirement of the growing rat and the nonessential amino acids were in the pattern of rat carcass.

The primary purpose of experiment 1 was to determine whether the observed difference in the need for glutamic acid could be related to the pattern of essential amino acids, to the pattern of nonessential amino acids, or to the relative proportion of essential to nonessential amino acid mixtures. This was investigated by interchanging the essential and nonessential amino acid mixtures as used in basal diets D and E. Three graded levels of the two nonessential mixtures (table 1) were combined with each of the two essential mixtures as given in table 2. The combination of level one of nonessential mixture D with essential mixture D provided a diet identical to basal diet D of the previous study (2). All combinations were fed without supplement and those with nonessential levels 1 and 2 were also fed with the addition of 4% of L-glutamine.

The results in table 3 show the dependency upon glutamine to be related to the nonessential amino acid pattern and spe-

[7] Unpublished data.

TABLE 1

Composition of nonessential amino acid mixtures

Amino acid	Nonessential mixture E[1]			Nonessential mixture D[2]		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
	%	%	%	%	%	%
L-Aspartic acid	1.50	2.33	3.16	0.70	1.10	1.49
L-Proline	0.83	1.29	1.75	0.39	0.61	0.83
Glycine	1.47	2.29	3.11	2.01	3.13	4.25
L-Alanine	0.99	1.54	2.09	0.44	0.68	0.92
L-Serine	—	—	—	0.92	1.44	1.95
(Nitrogen contribution)	(0.688)	(1.072)	(1.455)	(0.688)	(1.072)	(1.455)

[1] Relative proportions as found in rat carcass, except serine omitted.
[2] As contained in basal diet D of previous studies (2).

TABLE 2

Composition of essential amino acid mixtures

Amino acid	Mixture E [1]	Mixture D [2]
	%	%
L-Arginine·HCl	0.97	0.97
L-Histidine·HCl·H_2O	0.40	0.67
L-Isoleucine	0.66	0.71
L-Leucine	0.84	1.15
L-Lysine·HCl	1.35	1.37
L-Phenylalanine	0.50	0.84
L-Tyrosine	0.36	0.50
L-Methionine	0.25	0.47
L-Cystine	0.35	0.33
L-Threonine	0.60	0.51
L-Tryptophan	0.18	0.21
L-Valine	0.66	0.73
(Nitrogen contribution)	(1.015)	(1.170)

[1] Proportions based on requirement pattern, amino acids in 20% excess of requirement.
[2] As contained in basal diet D of previous studies (2).

cifically to nonessential mixture D. Application of the new Duncan multiple range test (shown by superscript notation in table 3) shows no significant difference between essential amino acid mixtures D and E, nor among levels of either nonessential mixture under a given set of conditions.

Growth rates and food efficiency ratios were severely depressed with all diets in which nonessential amino acid mixture D was fed without glutamine, but approached maximal values when glutamine was included in the diet. Nonessential mixture E produced the highest rates of gain when fed with glutamine but these were only slightly lower when glutamine was omitted and food efficiencies were unaffected.

Analyses were performed on urine samples collected from animals receiving diets containing the amino acid mixtures at the lowest levels. The results with essential mixture E diets are shown in table 4 for comparison with those obtained in experiment 2 (table 5). In the absence of glutamine, nonessential mixture D produced greater excretion of serine, glycine, threonine, histidine, taurine, hydroxyproline, alanine, lysine, glutamine/asparagine, and urea than did mixture E. When fed with glutamine these differences were eliminated or greatly reduced and the amounts of urea and ammonia were increased.

Expriment 2. Since performance did not depend upon the level of nonessential mixture fed in experiment 1, it appeared that the relative proportion of amino acids within the mixture was the more critical factor. A comparison of the two nonessential mixtures shows that the only major differences were in the proportions of glycine and serine. This is made especially obvious when level 3 of nonessential mixture D is compared with level 1 of nonessential mixture E (table 1). Experiment 2 was conducted to test whether the differences observed in experiment 1 were re-

TABLE 3

Comparison of amino acid mixtures on requirement for glutamine (exp. 1)

Nonessential amino acids			Essential amino acids			
			Mixture E		Mixture D	
Mixture	Level	Glutamine	Weekly gain [1]	Food efficiency	Weekly gain	Food efficiency
		%	g	g gain/ g food	g	g gain/ g food
E	1	0	41.0[abcd] ± 1.0	0.44	39.5[abcd] ± 1.4	0.44
	2	0	38.5[cd] ± 1.0	0.43	39.7[abcd] ± 1.7	0.45
	3	0	38.7[cd] ± 1.3	0.44	39.0[bcd] ± 1.5	0.45
	1	4	43.3[a] ± 1.6	0.43	42.4[abc] ± 1.9	0.43
	2	4	43.1[ab] ± 1.5	0.44	42.1[abc] ± 1.4	0.45
D	1	0	26.4[e] ± 1.2	0.37	25.7[e] ± 1.0	0.38
	2	0	23.0[e] ± 0.6	0.36	23.7[e] ± 0.8	0.38
	3	0	24.5[e] ± 0.8	0.38	24.7[e] ± 0.3	0.39
	1	4	39.3[abcd] ± 0.8	0.41	38.8[cd] ± 2.3	0.43
	2	4	36.5[d] ± 1.4	0.43	36.5[d] ± 1.0	0.43
Gluten control diet			50.2 ± 1.6	0.52		

[1] Average of 8 rats ± SE over 3-week period. Means bearing same superscript letter not significantly different (5% level) by multiple range test (Duncan, D. B., Biometrics, *11:* 1, 1955).

TABLE 4

Urinary excretion of nitrogenous compounds as affected by the nonessential amino acid mixture and by addition of glutamine (exp. 1)

Diet description:				
Essential amino acids	Mixture E		Mixture E	
Nonessential amino acids	Mixture D		Mixture E	
Glutamine, % of diet	—	4	—	4
	µmoles excreted/100 g body wt [1]			
Taurine	16.3	3.7	8.1	5.0
Urea	716.0	1246.0	640.0	868.0
Hydroxyproline	1.2	0.4	0.8	1.0
Threonine	9.0	2.6	3.8	2.6
Serine	9.7	3.6	1.2	0.9
Glutamine (+ asparagine)	2.0	2.1	1.3	2.2
Proline	1.6	1.6	2.0	4.3
Glycine	23.1	6.0	5.9	4.7
Alanine	1.8	1.3	1.3	1.4
Ammonia	79.0	111.0	73.3	96.3
Lysine	2.8	2.2	2.2	2.3
Histidine	0.5	0.3	0.2	0.3
Creatinine	15.3	17.9	15.7	14.0
Arginine	1.0	1.0	0.7	1.2

[1] Average of duplicate analyses of pooled samples. Urine samples collected from 3 or 4 animals/group over 20-hour periods during third week of experiment.

TABLE 5

Urinary excretion of nitrogenous compounds as affected by dietary glycine and serine and by the addition of glutamine (exp. 2)

Dietary level, %								
Serine	—	—	1.95	1.95	—	—	1.95	1.95
Glycine	—	—	—	—	4.25	4.25	4.25	4.25
Glutamine	—	4.00	—	4.00	—	4.00	—	4.00
	µmoles excreted/100 g body wt [1]							
Taurine	2.1	1.9	0.9	3.6	20.3	10.8	21.8	9.9
Urea	229.0	665.0	430.0	1225.0	873.0	948.0	1141.0	1822.0
Hydroxyproline	0.8	0.4	0.6	1.0	3.2	1.7	3.4	1.3
Threonine	2.8	2.9	2.6	2.2	30.3	3.8	37.3	9.0
Serine	0.5	0.7	3.9	3.9	30.3	2.7	94.0	23.7
Glutamine (+ asparagine)	1.5	3.2	1.3	1.7	12.3	3.2	17.1	5.9
Proline	0.8	0.6	0.9	1.1	1.7	2.4	2.6	2.2
Glycine	1.5	1.9	2.7	2.0	437.0	23.4	554.0	81.3
Alanine	1.2	1.4	1.6	1.4	6.4	2.6	6.6	3.0
Ammonia	51.0	73.7	48.7	64.9	66.2	71.6	119.3	130.2
Lysine	1.8	2.0	1.9	1.7	4.3	2.4	4.3	2.6
Histidine	0.4	0.5	0.3	0.3	3.6	0.7	5.0	1.6
Creatinine	21.7	16.6	14.2	17.3	17.5	16.7	15.7	14.6
Arginine	0.9	0.8	1.2	0.8	1.0	2.0	1.6	2.2

[1] Average of duplicate analyses of pooled samples. Urine collected from 3 or 4 animals per group over 20-hour periods during third week of experiment.

lated to the relative proportions of glycine, serine, or their combined amounts in nonessential mixtures D and E.

Aspartic acid, proline, and alanine were maintained at the amounts shown for level 1 of nonessential mixture E (table 1) and were fed with essential mixture E (table 2). Increments of glycine (0, 1.47, and 4.25%) and serine (0, 0.86, and 1.95%) were tested separately and in combination with each other. The levels were chosen to include as nearly as possible the extremes of concentrations used in experiment 1 (nonessential mixture E, level 1; nonessential mixture D, level 3). All test combinations were fed both with and without added glutamine.

Performance results in experiment 2 are given in table 6. The highest rates of growth and food efficiency ratios resulted from diets containing no serine or glycine and with those containing the least addi-

TABLE 6

Gain and efficiency of food utilization as influenced by glycine and serine levels and by the addition of glutamine (exp. 2)

Dietary level		Fed without glutamine		Fed with 4% glutamine	
Serine	Glycine	Weekly gain [1]	Food efficiency	Weekly gain	Food efficiency
%	%	*g*	*g gain/g food*	*g*	*g gain/g food*
—	—	39.1[abc] ± 1.2	0.40	40.0[ab] ± 1.6	0.41
—	1.47	38.6[abc] ± 1.2	0.42	38.6[abc] ± 2.2	0.41
—	4.25	29.1[fg] ± 1.3	0.37	37.9[bc] ± 1.3	0.42
0.86	—	40.1[ab] ± 1.1	0.40	42.3[a] ± 0.9	0.41
0.86	1.47	32.3[def] ± 0.6	0.39	39.9[ab] ± 1.1	0.41
0.86	4.25	26.0[gh] ± 0.9	0.36	35.7[cd] ± 1.5	0.40
1.95	—	35.3[cd] ± 1.2	0.40	39.0[abc] ± 0.9	0.41
1.95	1.47	30.4[ef] ± 0.4	0.37	38.2[bc] ± 1.1	0.41
1.95	4.25	23.0[h] ± 0.9	0.34	32.7[de] ± 1.0	0.40
Gluten control diet		46.9 ± 1.1	0.48	±	

[1] Average of 8 rats ± SE over 3-week period. Means bearing same superscript letter not significantly different (5% level) by multiple range test.

tion levels. As indicated by the results of the multiple range test (shown in table 6) statistically equivalent gains were obtained with those diets regardless of whether glutamine was included. In the absence of glutamine, further additions of either serine, glycine, or both amino acids progressively decreased gain and food efficiency. When fed with glutamine, however, inhibition was evident only at the higher combined concentrations of serine and glycine.

A more definitive picture of the relationship between serine and glycine with glutamine is revealed by multiple regression analysis of the gain data. By this treatment it is demonstrated that the amount of inhibition was linear with respect to either excess glycine or serine and that the combined effects were additive. The data are found to fit the general expression:

$$Y = A + b_1X_1 + b_2X_2$$

in which Y is the average gain; A, the intercept constant; X_1 and X_2, the concentrations of glycine and serine; and b_1 and b_2, their respective partial regression constants.

The above expression would be expected to apply only to those diets in which the combined glycine and serine levels are actually inhibitory. It is presumed that a certain, optimal amount of glycine or serine must exist at which the gain would be maximal. Gains for diets containing 0% glycine, 0% serine, and for the diet containing 0% glycine, 0.86% serine plus glutamine were less than predicted by the regression equations. These data were excluded from the calculations because of the probability that gains were lowered because of suboptimal concentrations of glycine or serine.

Regression analyses were performed separately for the group of diets without glutamine and for those containing glutamine. Results are shown in figure 1, in which the two regression equations are drawn as separate planes in the three-dimensional representation.

For diets without glutamine the partial regression constants were found to be − 9.43 for glycine and − 11.26 for serine. The multiple correlation coefficient (R) of − 0.996 was highly significant, as were each of the partial correlation coefficients: − 0.986 for glycine and − 0.996 for serine. The coefficient of determination ($100R^2$) was 99.3. This value indicates the percentage of the regression in gain accounted for by the combined effects of glycine and serine.

With diets containing added glutamine, partial regression constants of − 4.62 and − 7.45 were obtained for glycine and serine, respectively. For this set of data, the multiple correlation coefficient was −0.989 and partial correlation coefficients were − 0.971 and − 0.988 for glycine and serine, respectively. All correlation coefficients were highly significant. The coefficient of determination in the presence of glutamine was 97.9.

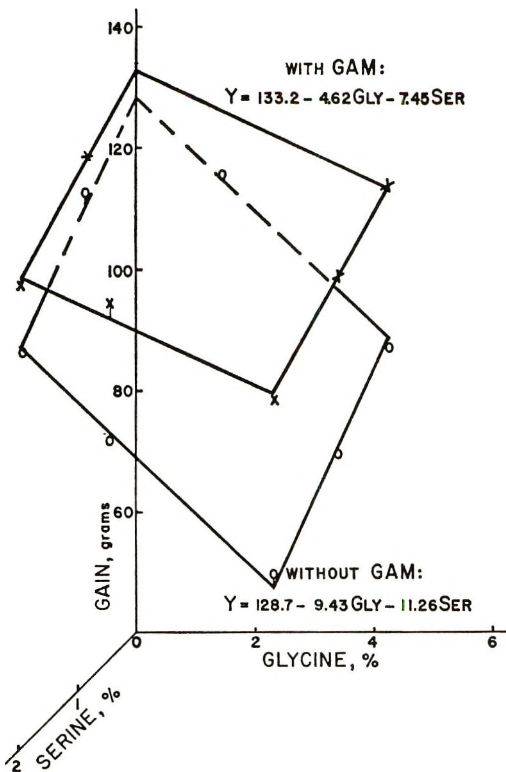

Fig. 1 Multiple regression planes calculated from 3-week gain data of experiment 2, showing experimental observations of gain in absence of glutamine (o) and with diets containing 4% of glutamine (x).

The multiple regression equations clearly indicate that glutamine reduced the inhibitory effects of excess glycine and serine, but not equally, since the partial regression constants were not lowered in the same proportion.

The inhibitory effects of glycine and serine are well-described by the regression equations for the experimental conditions of experiment 2, in which additions were made to a constant level of other amino acids. The relationship must be more complicated than described, however, because in experiment 1, variation in glycine and serine, as components of the nonessential amino acid mixture, did not produce significant changes in growth rate. It may be that the inhibitory effects are related to the proportion of glycine and serine to one or more of the remaining amino acids.

Samples of urine were taken from those groups receiving the dietary extremes of glycine and serine, both with and without the addition of glutamine. Table 5 lists the results of analyses for those compounds found in concentrations of at least 1 μmole per 100 g body weight. They support and serve to explain the results in table 4. In comparison with urine from those receiving the diet containing neither glycine nor serine, the addition of glycine alone greatly increased urinary glycine and serine and to a lesser degree threonine, taurine, histidine, glutamine/asparagine, alanine, urea, hydroxyproline, lysine, and proline (proportionate basis). Fewer differences were produced by serine alone, with the increase in serine itself as the main effect and with lesser increases in glycine, urea and alanine. But when fed in combination with glycine, serine accentuated the excretion of threonine, glutamine/asparagine, histidine, proline, and ammonia. The inclusion of glutamine in the diet greatly reduced the effects of glycine and serine and elevated the excretion of urea and ammonia.

DISCUSSION

The results presented here are of interest both from the aspect of the inhibitory effects of glycine and serine and from that of the role of glutamine in their counteraction. The nonessential amino acids have been considered to be generally noncritical for the rat. Stucki and Harper (3) reported uniformly good results over a range of indispensable-to-dispensable amino acid ratios of from 4 to 1. The results of experiment 1 indicate that altering the proportion of the nonessential amino acid mixture had little influence on growth rate, but those from both experiments illustrate the importance of the relative proportions of amino acids within the nonessential mixture. Others have noted inhibitory effects of glycine and serine, but under more extreme dietary conditions. When fed as the sole source of nonessential nitrogen glycine was found to be poor and serine ineffective by Rechcigl et al. (4), and Greenstein and Winitz (5) reported serine to be growth-depressing. When tested separately at 5% levels with a low protein

diet, Sauberlich (6) reported a 50% depression of weight gain with glycine but only 16% with DL-serine. He noted that the degree of toxicity of amino acids in excess was decreased at higher protein levels and was influenced by the type of protein fed. Gains were nearly 90% of control values when glycine was fed with 10% of casein, blood fibrin, or soy protein but only 53% with lactalbumin and 63% with egg albumin. Sauberlich also observed that glycine was unique among amino acids tested for toxicity in that the decrease in growth rate was not accompanied by lowered food consumption, thus resulting in lowered food efficiency. The decrease in food efficiency ratios with increments of glycine and serine in the present study supports this observation. The growth depression does not appear to operate through an appetite control mechanism such as suggested by Sanahuja and Harper (7) in their studies with amino acid imbalances.

Excretion in the urine of large amounts of glycine and serine as a result of their separate and combined additions to the diet indicates their close metabolic relationship and suggests an inadequate ability of the rat to metabolize or detoxify these amino acids under the dietary conditions. The increased excretion of other amino acids may reflect altered patterns of metabolism or changes in rates of reabsorption. Handler et al. (8) observed that glycine infused in dogs increased the excretion of threonine and histidine primarily, and Greenstein and Winitz (5) considered it likely that the renal threshold is low for threonine, histidine, and lysine.

The accumulation of threonine could possibly arise from high plasma concentrations of serine because Goldstein et al. (9) found both substances to have a common dehydratase which is more active for serine than threonine and which is not activated by threonine. Anderson and his associates[8] reported the activity of this enzyme to be greatly increased by feeding high protein diets and it may be that in the present experiments the nitrogen level was too low to provide adequate stimulus.

It is not certain whether the influence of glutamine in counteracting the effects of glycine/serine is general or specific in nature. It remains to be determined whether it facilitates their normal metabolism or whether it provides a process of detoxification.

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Effect of Method of Fluoride Administration on Plasma Fluoride Concentrations [1]

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ABSTRACT Two groups of sheep were given 2 mg F/kg either as a single oral dose or as a continuous intrarumenal infusion for a 13-week period, and the response in plasma fluoride was determined. After an oral dose there was a rapid increase in plasma fluoride to a peak at 2 to 3 hours and a slower decline to near the predosage level by 12 hours. A considerable difference in the manner in which individual sheep metabolized fluoride was evident from the daily plasma fluoride curves. When the same amount of fluoride was continuously infused, the plasma fluoride concentration was relatively constant throughout the day, and except for the peak period of about 6 hours, was higher than that from the sheep given an oral dose.

The gross responses of laboratory and domestic animals to the ingestion of elevated amounts of fluoride in the diet have been extensively studied and reviewed (1–3). The concentration of fluoride in normal plasma is low and is usually found to increase only slightly following fluoride ingestion. From data obtained in both rats and humans, Singer and Armstrong (4, 5) have postulated that there is a homeostatic mechanism which maintains this low level of plasma fluoride. However, Shearer and Suttie (6) have shown that when rat plasma is sampled soon after the consumption of a diet containing elevated concentrations of fluoride, there are significant increases in plasma fluoride. Muhler et al. (7) have recently demonstrated rapid variations in plasma fluoride following the administration of a single oral dose to dogs and humans.

It has also been postulated (8) that the concentration of fluoride in plasma and other soft tissues will increase with time as the ability of the skeleton to retain additional fluoride diminishes and that this increase will be associated with an increased severity of fluoride-induced effects in the animal.

The objective of the present study was to determine the effect of a single dose, compared with a continuous infusion of fluoride on plasma fluoride concentrations and to determine what influence the duration of administration might have on these concentrations. The relative toxicity of fluoride administered by these 2 methods was also investigated by determining the net fluoride balance over a limited time-period and by analysis of skeletal fluoride retention.

METHODS

Nine wether lambs weighing about 50 kg at the beginning of the experiment were given 2 mg of fluorine (as NaF) per kg of body weight per day for a 3-month experimental period. Six of the nine received this dose in a gelatin capsule before feeding in the morning, and the other three received the same amount dissolved in distilled water and continuously infused into the rumen through an artificial fistula in the left flank.

Blood samples were taken from the jugular vein at zero, 0.5, 1, 2, 3, 5, 8, 12, and 24 hours after administration of the F capsules on days 1, 2, 8, 15, 22, 36, 50, 64, and 78 of the experiment, and plasma fluoride was determined by the method of Singer and Armstrong (9).

Biopsy samples were obtained from the hip bone under local anesthesia at the start of the experiment, and after 14 weeks of fluoride administration. The samples

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were dried, ashed, and analyzed for fluoride by the Willard and Winter method (10).

During a 5-day fluoride balance study, urine was collected twice daily, just before the oral F administration and 12 hours later. The amount of fluoride in each sample was determined by the method of Rowley and Farrah (11) modified by the use of the color reagent of Singer and Armstrong (9). Fecal samples from each sheep for the entire 5-day study were collected, mixed, and a representative sample was analyzed for fluoride.

The sheep were fed a mixture of ground alfalfa hay (79.5%), cracked corn (10%), whole oats (10%) and trace mineralized salt (0.5%) immediately following the zero hour sample at 8:30 AM each morning. Water containing 1 ppm F was supplied ad libitum.

RESULTS

The diurnal changes in plasma fluoride concentrations which were evident over the nine daily sampling periods for each of the 6 sheep in the group given a single daily dose of fluoride are shown in figure 1. These data indicate there was a rapid increase in plasma fluoride following administration of the 2 mg F/kg dose, and that following a peak which usually occurred within 3 hours there was a slower decrease to nearly preingestion values by 12 hours.

The data in figure 1 also indicate there can be rather marked differences in the ability of individual animals to handle fluoride. These data, which are the average for each time-period for each animal during the last 5 sampling periods (22–78 days on experiment), show that sheep A consistently responded to fluoride ingestion with a more rapid and extensive increase in plasma fluoride and that the plasma peak was reached earlier in this animal than in the others. Sheep B also had a higher plasma fluoride peak, whereas the remaining 4 animals exhibited practically identical plasma patterns. To illustrate the consistency of the diurnal pattern of each sheep from one sampling period to another, the actual plasma fluoride values for two of these sheep and one of the continuously infused animals are presented in table 1. The rather small variations observed in the time at which the plasma fluoride peaked and the actual plasma fluoride level of an individual sheep at different sampling periods indicate that the variations between the animals (fig. 1) are a true reflection of variation in the absorption, distribution or excretion of fluoride between different animals.

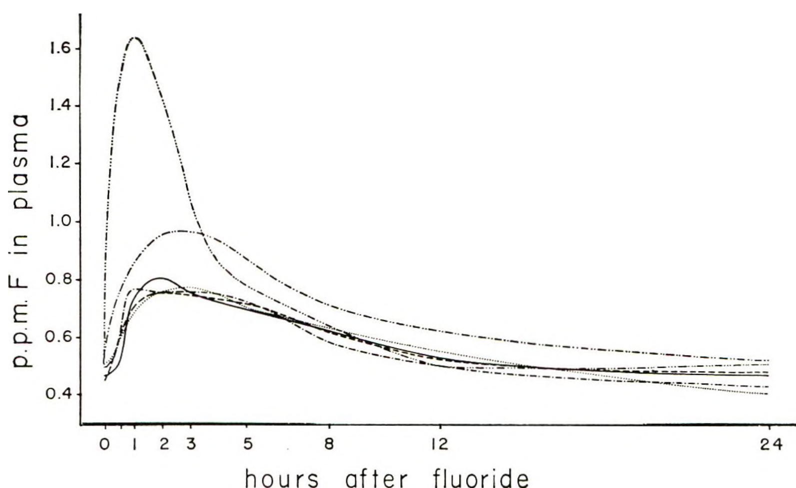

Fig. 1 Diurnal variation in plasma fluoride. Each curve represents a single animal given an oral dose of 2 mg F/kg at zero time. The plasma fluoride value for each time-period is the average of the last five sampling periods. The animals referred to in the text or in table 1 are: A, — · · · ; B, — · · ; and C, — · —.

TABLE 1

Diurnal variation in plasma fluoride [1]

Animal and treatment	Days on experiment	F in plasma at hour								
		0	0.5	1	2	3	5	8	12	24
		ppm	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
A Single dose	22	0.60	0.80	2.26	1.80	1.75	1.26	1.04	0.52	—
	36	0.63	2.26	1.96	1.69	0.59	0.65	0.67	0.50	0.38
	50	0.58	2.41	2.01	1.37	1.24	0.83	0.65	0.53	0.50
	64	—	1.44	1.43	1.59	1.04	0.56	0.42	0.39	0.39
	78	0.44	1.45	1.18	1.43	1.10	0.67	0.43	0.41	0.58
C Single dose	22	0.48	0.46	0.70	0.88	1.01	0.76	0.97	0.56	0.31
	36	0.55	0.65	0.71	0.85	0.73	0.63	0.65	0.55	0.51
	50	0.52	0.63	0.68	0.71	0.81	0.72	0.65	0.65	0.31
	64	0.48	0.56	0.49	0.69	0.76	0.62	0.59	0.51	0.47
	78	0.54	0.63	0.54	0.64	0.65	0.70	0.62	0.46	0.36
D Continuous infusion	22	0.73	0.71	0.81	0.69	0.68	0.63	0.55	0.61	0.42
	36	0.63	0.76	0.64	0.77	0.76	0.54	0.80	0.72	0.96
	50	0.78	0.67	0.86	0.69	0.74	0.73	0.72	0.76	0.59
	64	0.70	0.68	0.79	0.69	0.63	0.55	0.45	0.41	0.48
	78	0.68	0.72	0.57	0.71	0.57	0.53	0.56	—	0.46

[1] See figure 1 for identification of animals A and C, and figure 2 for the diurnal curve of the continuous group of which animal D is an example.

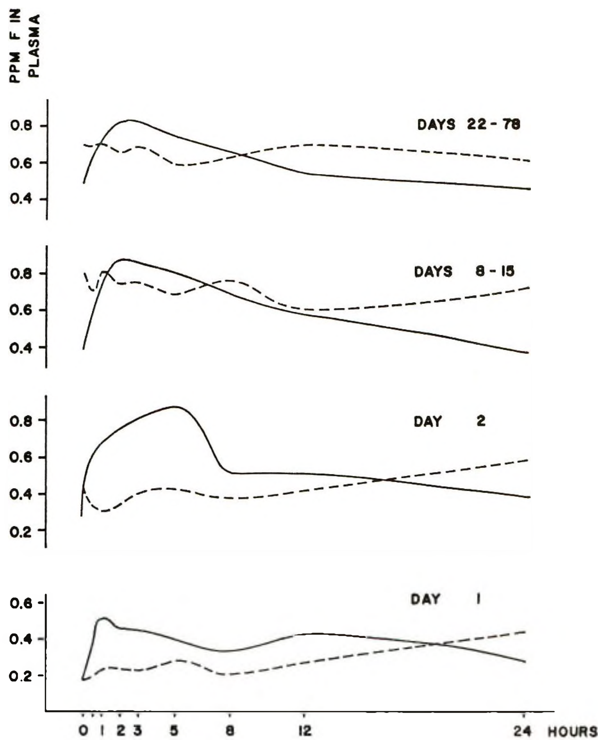

Fig. 2 Changes in the diurnal plasma fluoride pattern with an increasing period of exposure. The solid line is the curve for the 6 sheep given an oral dose of fluoride, and the dashed line the curve for the 3 sheep given a continuous infusion of fluoride. Each curve is an average of all sheep in the group for the periods of exposure indicated.

That the extent of previous fluoride exposure has some influence on the animals, metabolism of fluoride is illustrated in figure 2. The plasma fluoride concentration was about 0.2 ppm before fluoride administration was begun, and the "zero time," or 8:30 AM, plasma level increased to about 0.4 ppm F by the end of 2 weeks of a single daily oral dose of fluoride. This value was increased only slightly during the remainder of the experiment. There was a rather constant increase in the concentration of plasma fluoride in the continuously infused sheep over the first 48 hours to a level of about 0.6 ppm F. Following this, the values increased to about 0.7 ppm by the end of the second week, and there was little further increase during the remainder of the 13-week experiment. After the first few days, the plasma fluoride level of the sheep continuously infused with fluoride was higher at zero time, fell below that of the animals given a single dose for about 8 hours, and then was higher again for the remainder of the day. The sheep were fed following the zero-time sample, and the variations in plasma F concentrations noted in the infused sheep during the subsequent few hours were probably related to the introduction of food into the rumen.

The data from the balance study (table 2) indicate that about 60% of the fluoride administered each day was excreted and that there was no statistically significant difference in retention between the 2 groups. There was, however, an apparent increase in the amount of fluoride which had been absorbed from the rumen in the infused group. This is evidenced by the increase in urinary and decrease in fecal excretion in this group compared with the orally administered group. As was to be expected, the urinary fluoride concentration in the infused group was rather constant and averaged about 50 ppm (corrected to specific gravity = 1.040) during both the day and night collection period. The sheep given a single oral dose in the morning excreted urine containing about 49 ppm F during the day and only 14 ppm during the night. Fluoride analyses of the bone biopsies showed that although the skeletal retention was similar in both groups, there was a slightly higher increase in skeletal fluoride when the fluoride was administered by constant infusion. The fluoride content of the hip bone at the beginning of the experiment was 443 ± 41 ppm (ash weight) and after 14 weeks was 5935 ± 213 ppm in the 6 sheep given an oral dose, and 6763 ± 129 ppm in the 3 sheep continuously infused with the same amount of fluoride per day.

TABLE 2

Fluoride balance — days 38–42

	Continuous infusion	Oral dose
F intake, mg	524 ± 40 [1]	558 ± 24
F excretion, mg		
Fecal	119 ± 31	186 ± 38
Urinary	197 ± 14	123 ± 9
F retained, mg	208 ± 17	249 ± 35
F retention, %	40 ± 4	45 ± 6

[1] Values are mean ± SE for the 3 continuously infused sheep, and 3 of the 6 sheep given an oral dose of fluoride.

DISCUSSION

These data confirm previous reports which indicate a rapid but transient increase in plasma fluoride following an oral dose of a soluble fluoride (7), and extend them to indicate that this diurnal variation occurs even after a prolonged period of ingestion which has resulted in appreciable skeletal retention of fluoride. The variation in the plasma fluoride response of different animals to the same fluoride dose was particularly marked. The reason for this individual difference was not determined, but it is clear from the reproducibility of pattern at different weeks that it was a true difference in the way in which different animals metabolized fluoride. Whether this difference was due to a variation in rate of absorption or excretion of fluoride cannot be determined from the data. Individual differences such as these might be responsible for some of the variation in the degree of dental damage noted in animals fed identical amounts of fluoride experimentally, or in animals which appear, on the basis of skeletal fluoride concentrations, to have been exposed to similar amounts of fluoride (12).

Inspection of these data shows that although the plasma fluoride concentration decreased rapidly from a peak obtained after a single dose, it did not decrease

to the pre-experimental value of about 0.2 ppm but remained somewhat elevated for the remainder of the day. This baseline plasma fluoride level did not increase appreciably following the first two or three weeks, indicating that even though there had been a large increase in skeletal fluoride, the ability of the animal to rapidly clear fluoride from the plasma had not been impaired. After the first two or three weeks, the continuous infusion of an identical amount of fluoride was found to maintain a higher concentration of plasma fluoride for more total hours during the day than did the oral dose, even though the peak value was lower. These data, however, do not allow an interpretation of which treatment might eventually be more damaging to the animal, the high peak value from the oral administration or the extended period of slightly elevated fluoride during the day from continuous infusion.

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Depression of Lipogenesis in Choline-deficient Guinea Pigs and its Possible Mechanisms

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ABSTRACT Feeding guinea pigs a choline-deficient diet resulted in marked changes of cell constituents and their phospholipid composition, indicating that the animals have only a small capacity for synthesis of choline. Such changes were linked with depression of lipogenesis and inhibition of glucose permeability. When choline chloride was injected into the deficient animals, phospholipid synthesis was greatly increased. The inhibition of glucose permeability was marked in adipose tissue. Adipose tissue of choline-deficient animals did not respond to insulin, but treatment with choline chloride reactivated the response. Possibly a decrease of phospholipid synthesis and an insufficient supply of glucose in choline deficiency results in accumulation of free fatty acid or acyl-CoA, which inhibits fatty acid synthesis. After choline administration, an increase in the incorporation of fatty acids into phospholipid and the activation of glucose permeability might stimulate synthesis of fatty acids. The levels of intracellular glucose or glucose metabolite would then be related to triglyceride synthesis and secondly to fatty acid synthesis.

In a previous study in this laboratory, guinea pigs fed a choline-deficient diet showed marked growth disturbance, and young animals died within 4 to 5 weeks; also the content of various choline derivatives in cell constituents decreased (1). It was postulated that guinea pigs have a low capacity for the synthesis of choline (2), and that a choline deficiency might cause changes in cell structure through a disturbance in the metabolic turnover of choline derivatives such as lecithin, and inevitably lead to malfunctioning of the cells. One of the effects observed was the significant depression [1] of D-β-hydroxybutyric dehydrogenase (3). The intraperitoneal injection of choline chloride overcame such effects of choline deficiency at every stage of the experiment, and the rate of body weight gain was significantly increased by the injection.

A low level of liver glycogen and poor storage of carcass lipid have been noted in choline-deficient guinea pigs. The content of lipid in the liver was also slightly decreased, in contrast with the results for rats (4). It was suggested that a disturbance of intestinal absorption of nutrients may result in a low level of liver glycogen, hypoglycemia, and depressed lipogenesis. However, the changes in cell constituents may also be the direct effect of choline deficiency in addition to a disturbance in intestinal absorption. To clarify this question, the utilization of ^{14}C-U-glucose was analyzed under various conditions both in vivo and in vitro. These results suggested a depression of glucose transfer across the cell membrane in the animals fed a choline-deficient diet. The mechanism whereby choline deficiency depresses lipogenesis based on this feeding is discussed in the present paper.

EXPERIMENTAL

Animals and treatments. Except for the experiments on utilization of ^{14}C-U-glucose, male guinea pigs weighing 250 g were used. The animals were fed for 3 weeks the diet [2] listed in table 1, following a 3-day adaptation period during which the diet was supplemented with vegetables. In the experiment as shown in tables 2 and 3, the animals weighing 180 to 200 g, were fed the diet for 4 weeks. Under these conditions the effects of a choline deficiency in the diet were readily developed as reported previously (1). In the assay

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[1] Presented at a meeting of Vitaminology, Japan, 1966.

[2] 0.2% choline chloride was added to the control diet.

TABLE 1
Composition of diet [1]

	%
Vitamin-free casein [2]	29.12
Sucrose	10.00
Starch	19.42
Cellulose	7.57
Cellophane	14.56
Rapeseed oil	7.09
Salt mix [3]	5.83
Potassium acetate	2.43
Magnesium oxide	0.49
Vitamin mix [4]	3.30
Vitamin C	0.19

[1] Ten milligrams of folic acid, 100 mg of vitamin E (20% α-tocopherol) powder and 700 mg of vitamin A, D mix were added to 100 g of the diet. One gram of the A, D mix contained 50,000 IU of vitamin A and 10,000 IU of vitamin D_2.

[2] Obtained from Takeda Chemical Corporation, Nagoya, Japan.

[3] Percentage composition of the salt mix was: $CaCO_3$, 29.29; NaCl, 25.06; $ZnCl_2$, 0.02; KI, 0.0005; $CaHPO_4 \cdot 2H_2O$, 0.43; $MgSO_4 \cdot 7H_2O$, 9.98; $CuSO_4 \cdot 5H_2O$, 0.156; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0025; KH_2PO_4, 34.31; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.623; and $MnSO_4 \cdot H_2O$, 0.121 (obtained from Tanabe Pharmacal Corporation, Osaka, Japan).

[4] Percentage composition of the vitamin mix was: thiamine·HCl, 0.059; riboflavin, 0.059; nicotinic acid, 0.294; Ca pantothenate, 0.235; pyridoxine·HCl, 0.029; menadione, 0.006; biotin, 0.001; folic acid, 0.002; vitamin B_{12}, 0.0002; inositol, 1.176; ascorbic acid, 0.588; and lactose, 97.551 (obtained from Tanabe Pharmacal Corporation).

for utilization of the isotopic compounds in vivo, 7.6 μCi of ^{14}C-1-acetate or 8 μCi of ^{14}C-U-glucose were administered intraperitoneally, in 1 ml of 0.9% NaCl solution or with 150 mg of glucose. The expiratory CO_2 was trapped in 70 ml of 2.5 N NaOH for one hour, using a metabolic cage. In this case, CO_2 and $^{14}CO_2$ were measured as the barium salt. After CO_2 measurement, the animals were killed by cervical section and exsanguinated and the liver was resected quickly. For the assay of ^{14}C-choline incorporation into lipid, the animals were fed for 2 weeks the same diet, and ^{14}C-choline was injected intraperitoneally with 40 mg of choline chloride and 150 mg of glucose.

Preparation for analysis. Liver lipid was extracted in 19 volumes of a chloroform-methanol (2:1, v/v) mixture by the method of Folch et al. (5). One gram of the liver, after homogenization with the above solvent mixture in a Waring Blendor, was extracted overnight at room temperature in a 100-ml glass-stoppered vessel and the volume was adjusted to 50 ml with the same solvent. Twenty milliliters of the extract were washed once with 4 ml of 0.04% $CaCl_2$ and then 3 times with a chloroform-methanol-water (3:48:47, v/v) mixture. After washing, the solvent was evaporated and the extract was analyzed. Liver fractionation was performed according to the method of Schneider and Hogeboom (6). For determination of carcass lipid, the frozen carcass was minced and then prepared by the method used for liver. For analysis of plasma the heparinized blood was used. For separation of lipid silicic acid, column chromatography was performed by the method of Rhodes and Dawson (7).[3] Liver glycogen was determined by the anthrone method (8).

Determination of CO_2 and $^{14}CO_2$ in the experiments in vivo. Seventy milliliters of 2.5 N NaOH, into which expiratory CO_2 was trapped, were diluted to 200 ml with distilled water. One part of the diluted solution was sedimented by adding an excess of a solution which contained 0.4 N NH_4Cl and 0.5 N $Ba(OH)_2$. The $BaCO_3$ sediment was washed 4 times with distilled water and dried. It was then weighed and counted.

Isotopic measurement and in vitro assay of ^{14}C-incorporation. The lipid extract of both liver and carcass, and $BaCO_3$ were counted by a 2 π gas flow counter [4] or by a Packard Tri-Carb liquid scintilation counter. For the assay of ^{14}C-incorporation in vitro, 500 mg of liver slices or 100 mg of epididimal adipose tissue taken from animals fed ad libitum the diet listed in table 1 were used. According to the method of Madson et al. (9) the reaction was performed in a rubber-stoppered vessel which contained 5 ml of Krebs-Ringer bicarbonate buffer (pH 7.4), 5 mM glucose, 2 mM acetate and 0.5 μCi of ^{14}C-1-acetate or ^{14}C-U-glucose at 37° under the gas phase CO_2–O_2 (5% CO_2–95% O_2). After 1.5 or 3 hours the reaction was stopped by adding 0.2 ml of 5 N H_2SO_4. The CO_2 formed was absorbed by 1 ml of ethanolamine poured into the center well through the rubber stoppers. Ethanolamine solution was counted as $^{14}CO_2$ formed. To the reaction mixtures, KOH was added to 2 N

[3] The lipid was extracted from 2 g of liver and washed by the method of Folch et al. (5); it was fractionated by column chromatography on 10 g of silicic acid and separated into fractions of 150 ml of $CHCl_3$ and 100 ml of MeOH.

[4] Kobe Kogyo Corporation, Japan.

TABLE 2

Effects of feeding a choline-deficient diet on guinea pigs [1]

Animal no.	Body wt			Duration	Dietary intake [2]	Liver Wt	Liver glycogen [3]	Liver lipid [3]	Carcass lipid [3]	$BaCO_3$ formed [4]
	Final	Initial	Gain							
	g	*g*	*g*	*days*	*g/day*	*g*	*mg/g liver*	*mg/g liver*	*mg/g carcass*	*mg/g body wt*
					Control					
1	375	174	201	30	28	16.8	15.8	42.8	63.2	12.06
2	377	206	171	30	28	17.9	31.3	41.6	69.5	8.96
3	295	204	91	28	20	12.9	40.2	40.4	51.6	11.88
4	335	195	140	28	24	15.1	16.4	42.6	68.3	11.24
5	269	176	93	28	20	11.4	18.8	40.2	55.8	11.92
Mean							24.5 ± 6.88 [5]	41.5 ± 1.06	61.7 ± 6.82	11.21 ± 1.14
					Deficient					
1	250	208	42	30	20	6.85	1.4	37.6	22.5	10.92
2	200	194	6	30	18	9.06	30.0	35.2	31.0	14.52
3	211	176	35	30	14	7.10	1.0	36.4	20.0	11.68
4	188	166	22	28	16	6.80	12.5	37.6	23.4	12.72
5	208	182	26	28	16	6.11	10.0	35.0	25.0	11.76
Mean							11.0 ± 10.38	36.4 ± 1.10	24.4 ± 0.66	12.32 ± 1.21

[1] The animals were fed 4 weeks ad libitum on the diet listed in table 1.
[2] Twenty-eight grams of the diet were given for the last 3 days.
[3] Difference between the 2 groups was significant ($P < 0.01$).
[4] Formation of expiratory CO_2 for one hour was represented as the weight of $BaCO_3$.
[5] Mean ± SE.

TABLE 3

The ^{14}C-incorporation to expiratory CO_2, liver lipid and carcass lipid for an hour after the administration of ^{14}C-1-acetate [1]

Animal no.	Calculated $^{14}CO_2$ formed [2]	Calculated ^{14}C-uptake to liver lipid	Calculated ^{14}C-uptake to carcass lipid [2,3]
	cpm/g body wt	*cpm/g liver*	*cpm/g carcass*
		Control	
1	10,380	203	115
2	9,210	246	194
3	8,214	222	151
4	9,860	333	205
5	11,480	249	209
Mean	$9{,}829 \pm 1{,}082$ [4]	251 ± 43.6	175 ± 35.6
		Deficient	
2	12,540	443	131
3	13,380	213	80
4	8,520	328	94
5	13,700	205	79
Mean	$12{,}035 \pm 2{,}070$	297 ± 186.0	96.0 ± 21.2

[1] 7.6 μCi of ^{14}C-1-acetate were injected intraperitoneally with 1 ml of 0.9% NaCl solution.
[2] The results were obtained from animals which were administered ^{14}C-1-acetate at a level of 7.6 μCi/200 g body weight.
[3] Difference between the 2 groups was significant ($P < 0.01$).
[4] Mean ± SE.

concentration and hydrolyzed in a boiling water bath for 2 hours. After adjusting the pH to 1.0, the hydrolysate was extracted for 15 minutes with 15 ml of petroleum ether using a mechanical shaker and the extractions were performed successively 4 times and the whole extract was washed by 1.5 N acetate and then by water until the aqueous layer became neutral. The extracts were counted with a scintillation counter, using the method of Jeffay and Alvarez (10) for $^{14}CO_2$ and by the method of Numa et al. (11) for fatty acid. The amount of fatty acid was determined by the method of Duncomb (12). The ^{14}C-choline, taken up into the lipid, was counted by the method of Numa et al. (11) after preparation of the lipid fraction according to Folch et al. (5). Bovine crystal insulin [5] was used for insulin preparation and was added to 0.2% gelatin-Krebs-Ringer bicarbonate buffer (13).

RESULTS

The animals were fed for 4 weeks a choline-deficient diet. Following this, a limited amount of diet was given to the control group for 3 days before the assay to establish the blood sugar levels in both groups, since the liver glycogen content in the choline-deficient animals decreased significantly when compared with that in the controls as reported previously (1). Table 2 shows the gain of body weight, liver weight, liver glycogen and carcass lipid. The results agree with our previous data except for liver glycogen level. The amount of the diet was sufficient for the deficient group. The liver lipid content in the deficient group decreased slightly and carcass lipid content in the deficient animals was much lower when compared with the control. These results were different from those obtained for rats (4). Under such conditions, the liver glycogen content of the control group was slightly lower than that of the controls in the other experiments. Nevertheless, liver glycogen content in the deficient group was lower than the control with one exception. Despite this condition there was a marked difference between the 2 groups in the ^{14}C-incorporation into carcass lipid from ^{14}C-1-acetate as shown in table 3. In tables 2 and 3 are also listed the values of expiratory CO_2 and $^{14}CO_2$ after administration of ^{14}C-1-acetate. $^{14}CO_2$ and CO_2 per unit weight in the deficient group was slightly higher, but the difference was

[5] Novo, Denmark; 25 U/mg.

TABLE 4

(A) *Incorporation of ^{14}C-U-glucose into carcass and liver lipids and fatty acids* [1]

	Body wt	Total carcass lipid [2]	^{14}C-incorporated into lipid [2]	^{14}C-incorporated into fatty acids [2]
	g	*mg*	*cpm/g carcass*	*cpm/g carcass*
		Carcass		
Deficient	233±4 [3]	4542±528	770±126	377±42
Injected [4]	245±3	7174±1253	1641±324	1203±353
Control [5]	231±4	24300±1968	1970±269	897±246

	Liver wt [2]	Total liver lipid [2]	^{14}C-incorporated into lipid [2]	^{14}C-incorporated into fatty acids [2]
	g	*mg*	*cpm/g liver*	*cpm/g liver*
		Liver		
Deficient	7.68±1.23 [3]	295.49±16.26	2790±163	755±146
Injected	12.36±1.56	388.92±12.48	4697±1023	3083±1074
Control [5]	13.76±1.46	474.38±20.52	3580±476	1434±22

(B) *Distribution of the ^{14}C in both chloroform and methanol fractions from ^{14}C-U-glucose*

	$CHCl_3$ fraction		F/G ratio [6]	MeOH fraction		F/G ratio
	Glyceride glycerol	Fatty acid		Glyceride glycerol	Fatty acid	
	cpm	*cpm*		*cpm*	*cpm*	
			Carcass			
Deficient	202±12 [7]	202±11 [7]	1.00	163±13	149±12 [7]	0.92
Injected	567±46	671±39	1.18	181±16	234±15	1.29
Control	532±24	696±26	1.31	239±17	259±21	1.08
			Liver			
Deficient	608±56	218±18 [2]	0.36	479±12 [2]	350±121 [2]	0.73
Injected	582±48	497±26	0.82	1255±21	1767±442	1.40
Control	890±58	597±17	0.67	627±14	626±132	1.00

[1] Eight microcuries of ^{14}C-U-glucose were administered intraperitoneally with 150 mg of non-radioactive glucose; each value is the mean of 5 animals.
[2] The difference among the 3 groups was significant ($P < 0.01$).
[3] Mean ± SE.
[4] Forty milligrams of choline chloride were injected for 3 days before the assay (once every day).
[5] The animals fed the control diet, and that had the same body weight as the deficient group, were used for the controls.
[6] Fatty acid-to-glyceride glycerol ratio.
[7] The difference between the deficient group and the others was significant ($P < 0.01$).

TABLE 5

Incorporation of ^{14}C-choline [1] *in the liver of guinea pigs fed for 2 weeks a choline-deficient diet*

Duration	Body wt	^{14}C into lipid in a whole cell	^{14}C into lipid in 105,000 *g* supernatant fraction	^{14}C into lipid in nuclei and cell particles	^{14}C into lipid in plasma	^{14}C into lipid in mitochondrial fraction	^{14}C into lipid in microsomal fraction
hr	*g*	*cpm/g liver*	*cpm/g liver*	*cpm/g liver*	*cpm/ml plasma*	*cpm/mg protein*	*cpm/mg protein*
3	266	16,666	1572	15,094	74	205	333
	300	14,456	1268	13,188	44	242	523
6	260	18,251	432	17,819	24	315	771
	270	18,814	344	18,470	42	363	733
20	266	8,034	140	7,894	21	75	187
	330	12,154	116	12,038	21	182	216

[1] 3 μCi of ^{14}C-choline were injected intraperitoneally with 40 mg of choline chloride and 150 mg of glucose.

small. The ^{14}C-incorporation into carcass lipid was significantly decreased in the deficient animals, whereas the incorporation to liver lipid was not significantly different in each group as shown in tables 2 and 3. As indicated in the table, only one animal in the deficient group had a high liver glycogen level and a relatively high incorporation of ^{14}C-1-acetate into liver lipid; even in this case the incorporation to carcass lipid was very low compared with the control.

However, such apparently high ^{14}C-incorporation in the liver of the deficient animals was not observed when 150 mg of glucose were administered at the same time (table 4). In this experiment, the animals were fed the diet ad libitum for 3 weeks. As shown in table 4, the ^{14}C-incorporation from ^{14}C-U-glucose to long-chain fatty acids was decreased in both liver and carcass of the deficient group, and the ^{14}C-incorporation into lipid was markedly different in each group. The ^{14}C-incorporation into fatty acids of the deficient group was depressed in both triglyceride and phospholipid fractions, whereas the ^{14}C-incorporation into glyceride-glycerol of triglyceride was much higher than that in the other groups only in the liver. On the other hand, the ^{14}C-incorporation into phospholipid in the injected group was markedly increased. Table 5 shows the incorporation of ^{14}C-incorporation of ^{14}C-choline into lipid in the liver of the choline-deficient animals. When 40 mg of choline were injected with 3 μCi of ^{14}C-choline and 150 mg of nonradioactive glucose, the ^{14}C-incorporation into lipid reached a maximal level 3 to 6 hours after the injection. The incorporation in both microsomal and mitochondrial fractions was much higher compared with that in the supernatant fraction. However, the concentration of ^{14}C-choline in plasma was very low.

To further clarify the above results in vivo, the ^{14}C-incorporation into long-chain fatty acids was analyzed using liver slices and adipose tissue. The experimental animals used in this assay were described above. Tables 6 and 7 represent the results; the incorporation of ^{14}C-1-acetate was markedly depressed in the deficient group even when the medium contained 5 mM glucose, 2mM acetate and 0.1 U/ml insulin. It was noted that this depression in adipose tissues was not improved by adding insulin. Also, when ^{14}C-U-glucose was used instead of ^{14}C-1-acetate, the same inhibition of ^{14}C-incorporation into fatty acid was observed in the deficient animals. This inhibition was also marked in adipose tissue though there was no marked inhibition in the liver.

Pretreatment of the deficient animals with choline chloride had a significant effect in increasing the uptake (tables 4, 6 and 7). In these cases 40 mg choline chloride were injected intraperitoneally once at 15 hours or 3 times for 3 days (once every day) before the assay. Though the presence of insulin in the medium had no effect on the uptake in the deficient animals, the incorporation into the animals receiving the pretreatment was accelerated by the addition of insulin and glucose.

On the other hand, the addition of choline chloride to the incubation medium had no effect on the increase of ^{14}C-incorporation into fatty acid in both liver slices and adipose tissue as shown in table 8.

Epididimal adipose tissues of both sides were used; insulin was added to the one preparation and not added to the other. As shown in table 9, samples taken from the deficient animals showed an inhibited ^{14}C-incorporation from ^{14}C-U-glucose and revealed no response to insulin. The control and the injected samples had a high incorporation and showed a marked response to insulin.

DISCUSSION

It was reported previously (1) that a disturbance of intestinal absorption of nutrients would be an important factor in the decrease of liver glycogen and carcass lipid. However, in addition to the efficiency of intestinal absorption, the utilization of absorbed nutrients and the reutilization of the storage lipid may also be considered as factors influencing the content of liver glycogen and carcass lipid. From such a point of view, the utilization of ^{14}C-U-glucose or ^{14}C-1-acetate was analyzed to clarify interrelations among several factors. The results presented here offer evidence that

TABLE 6

Incorporation of ^{14}C-1-acetate[1] into long-chain fatty acid (FA) and CO_2, in liver slices and adipose tissues

	Control		Deficient[2]		Injected[2,3]	
	CO_2	FA	CO_2	FA	CO_2	FA
	dpm/100 mg tissue	*dpm/μmole FA*	*dpm/100 mg tissue*	*dpm/μmole FA*	*dpm/100 mg tissue*	*dpm/μmole FA*
Adipose tissue						
No insulin	11,731 ± 2,578[4] (5)[5]	185.8 ± 59.2[6] (5)	8,594 (2) (13,517 ± 3,187)[7] (6)	31.9 (2) (40.2 ± 7.47)[7] (6)	22,066 (2)	346.0 (2)
Insulin	7,895 ± 1,523 (4)	191.1 ± 47.9[6] (4)	12,207 ± 1,100 (8) (9,366 ± 403)[7] (8)	4.6 ± 1.11 (8) (26.5 ± 1.81)[7] (8)	17,703 ± 2,465 (4)	412.5 ± 189 (4)
Liver						
No insulin	23,756 ± 1,605 (4)	38.9 ± 3.25[6] (3)	37,823 ± 1,470 (4)	5.1 ± 0.32 (4)	37,274 ± 1,750 (4)	42.9 ± 6.3 (4)
Insulin	23,492 ± 880 (4)	30.7 ± 3.33[6] (4)	41,254 ± 2,150 (4)	7.2 ± 0.68 (4)	36,918 ± 4,485 (4)	44.3 ± 3.02
	dpm/liver/ g body wt	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*
Liver						
No insulin	11,284 ± 762 (4)	4,620 ± 38.9[6] (3)	11,536 ± 448[8] (4)	940 ± 26.3 (4)	22,365 ± 210[8] (4)	7,000 ± 48.5 (4)
Insulin	11,159 ± 418 (4)		12,995 ± 645 (4)	985 ± 12.5[6] (4)	22,141 ± 498 (4)	4,970 ± 58.7 (4)

[1] 0.5 μCi of ^{14}C-1-acetate was added to the reaction medium; incubation time was 1.5 hours.
[2] As the weight of adipose tissue taken from the choline-deficient animals was small, each sample used for the assay was excised from 2 animals.
[3] Forty milligrams of choline chloride were injected intraperitoneally for 3 days (once every day) before the assay.
[4] Mean ± SE.
[5] Numbers in parentheses indicate number of animals/group.
[6] Difference between 3 groups was significant ($P < 0.01$).
[7] The animals in this group weighed 270 to 300 g at the start.
[8] Difference between the 2 groups was significant ($P < 0.01$).

TABLE 7

Incorporation of ^{14}C-U-glucose [1] into long-chain fatty acid (FA) and CO_2, in liver slices and adipose tissues

	Control		Deficient		Injected [2] 3 days [3]		Injected once 15 hr before [4]	
	CO_2	FA	CO_2	FA	CO_2	FA	CO_2	FA
	dpm/100 mg tissue	*dpm/μmole FA*	*dpm/100 mg tissue*	*dpm/μmole FA*	*dpm/100 mg tissue*	*dpm/μmole FA*	*dpm/100 mg tissue*	*dpm/μmole FA*
Adipose								
No insulin	27,656 ± 1,050 [5] (2) [7]	48.2 ± 19.2 [6] (2)			34,646(2)	173.0(2)		
Insulin	25,507 ± 5,150 (2)	48.7 ± 11.7 [6] (2)	23,051 ± 5,860(4)	12.0 ± 3.0(4)	32,430 ± 880(8)	284.3 ± 78.5(8)	26,076 ± 932(8)	31.6 ± 6.1(8)
Liver								
No insulin	4,734 ± 570(3)	2.96 ± 0.014(3)	5,774 ± 55(3)		6,064 ± 197(3)	2.68 ± 0.477(3)	6,748 ± 462(2)	
Insulin	4,446 ± 283(3)	2.87 ± 0.025(3)	6,064 ± 364(3)	3.93 ± 0.032(3)	6,140 ± 980(4)	4.46 ± 1.250(3)	8,730 ± 269(2)	
	dpm/liver/ g body wt	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*	*dpm/liver/ g body wt*
Liver								
No insulin	2,284 ± 24.3 [5,6] (3)	74.9 ± 5.4 [6] (3)	1,714 ± 1.7(3)	45.8 ± 4.7(3)	3,638 ± 12.2(3)	80.8 ± 6.8(3)	3,033 ± 32.5(2)	55.8 ± 5.3(2)
Insulin	2,112 ± 8.7 [6] (3)	61.6 ± 3.3 [6] (3)	1,819 ± 8.3(3)	35.9 ± 5.3(3)	3,684 ± 34.7(4)	108.8 ± 9.4(3)	3,924 ± 36.2(2)	65.4 ± 6.8(2)

[1] 0.5 μCi of ^{14}C-U-glucose was added to the reaction medium; incubation time was 3 hours.
[2] As the weight of adipose tissue taken from choline-deficient animals was small, each sample used for the assay was excised from 2 animals.
[3] Forty milligrams of choline chloride were injected intraperitoneally for 3 days (once every day).
[4] Forty milligrams of choline chloride were injected once at 15 hours before the assay.
[5] Mean ± SE.
[6] Difference between each group was significant ($P < 0.01$).
[7] Numbers in parentheses indicate number of animals/group.

TABLE 8

Effects of choline added to the incubation medium on the utilization of ^{14}C-U-glucose in adipose tissue taken from choline-deficient animals [1]

	Without choline	With choline [2]
	cpm/100 mg tissue	
$^{14}CO_2$ formed	3733 ± 324 [3]	3294 ± 423
^{14}C-incorporation into fatty acid	730 ± 82	690 ± 68

[1] Each value is the mean of 5 experiments.
[2] Forty mg/ml of choline chloride were added to the incubation medium.
[3] Mean ± SE.

TABLE 9

Utilization of ^{14}C-U-glucose and the effect of insulin on adipose tissue taken from guinea pigs fed a choline-deficient diet

	Without insulin	With insulin	Increase
	cpm/mg protein	*cpm/mg protein*	*cpm/mg protein*
	$^{14}CO_2$ formed		
Deficient	2437 ± 231 [2]	2225 ± 318	−212
Injected [3]	3526 ± 356	5452 ± 769	+1926
Control	5882 ± 826	7195 ± 924	+1313
	^{14}C incorporated into lipid		
Deficient	744 ± 108	996 ± 39	+222
Injected [3]	1841 ± 378	2598 ± 249	+657
Control	3771 ± 399	5364 ± 714	+1593

[1] The difference among 3 groups was significant ($P < 0.01$).
[2] Mean ± SE.
[3] Forty milligrams of choline chloride was injected intraperitoneally for 3 days before the assay (once each day).
[4] Each value was the mean of 5 experiments.
[5] Incubation medium contained 0.5 μCi ^{14}C-U-glucose, 10 mM glucose and with or without 100 milliunits/ml of insulin.

the effects of a dietary choline deficiency would be induced by the changes in cell constituents and that the composition of phospholipid might be associated with glucose permeability into cells.

Since the deficient animals tended to become hypoglycemic as described previously, the amount of the diet for the control group was limited or glucose was injected to establish the blood sugar level of the deficient group. Even in these cases lipogenesis was significantly depressed in the deficient animals. Also in the in vitro assay, the ^{14}C-incorporation from ^{14}C-1-acetate or ^{14}C-U-glucose into fatty acid in epididimal adipose tissue was depressed in the presence of 5 mM glucose, 2 mM acetate and 0.1 U/ml insulin. The depression was marked in adipose tissue in vitro and in carcass in vivo, whereas it was slight in the liver. In the animals receiving injections of choline chloride the depression of the ^{14}C-incorporation was significantly improved both in vivo and in vitro.

^{14}C-U-glucose incorporation into adipose tissue was significantly inhibited in the presence of 10 mM glucose in the deficient animals as shown in table 9. Choline treatment markedly reversed this depression. Furthermore, since the injected choline was incorporated into lipid in both microsomal and mitochondrial fractions at a maximal level within 3 to 6 hours, as shown in table 5, this effect of choline treatment could be attributed to the recovery of cell constituents, which was induced by feeding the choline-deficient diet. Possibly recovery of cell constituents overcame the depressed permeability. Rodbell (14) reported that treatment with phospholipase C changed glucose permeability of fat cells in vitro. This agrees with our results in the participation of phospholipid with glucose permeability. Furthermore, injections of choline elevated such depressed fatty acid synthesis from glucose or acetate in vitro, as shown in tables 6 and 7.

As shown in table 4 the ^{14}C-incorporation from ^{14}C-U-glucose in vivo was greatly inhibited, especially in carcass, whereas in the liver the ^{14}C-incorporation did not decrease as much, though that into fatty acid was depressed. However, the mechanism for this difference between liver and carcass is not clear at present. These results were observed also in vitro as shown in tables 6 and 7.

An injection of 40 mg of choline chloride into the deficient animals increased expiratory CO_2 formation about 15% compared with the controls. This observation also might substantiate a speculation that feeding a choline-deficient diet would bring about marked inhibition of glucose permeability into the tissues of guinea pigs.

As shown in table 8 addition of choline chloride to the incubation medium had no significant effect on the ^{14}C-incorporation in adipose tissue. Injections of choline chloride had a marked effect as shown in

all data, and most of the injected choline was incorporated into lipid in both microsomal and mitochondrial fractions; such effects of choline could be caused by phospholipid synthesis and by its incorporation into cell constituents. On the other hand, a characteristic observation in the injected group was the highly increased ^{14}C-incorporation into the phospholipid fraction, as shown in table 4. These results suggest that phospholipid synthesis of the deficient animals was markedly increased by injection of choline and that a large amount of fatty acid was incorporated into phospholipid along with its synthesis. The decrease of accumulated FFA would stimulate further synthesis of fatty acid (15, 16).

Accumulation of fat in the liver may be related to the intracellular supply of glucose. When the glucose supply is sufficient, intracellular FFA might accumulate as triglycerides; therefore in such cases lipogenesis would not be inhibited by the increased FFA. However, in guinea pigs, fat accumulation in the liver was not observed even when the animals were fed a choline-deficient diet.

The effect of insulin on lipogenesis has been reported by many investigators. Some of them postulated that α-G-P was a key intermediate (17) to lipogenesis and that insulin stimulates its formation. Addition of insulin increased $^{14}CO_2$ formation and ^{14}C-incorporation into lipid as shown in table 9. Under the same conditions adipose tissue taken from the deficient animals gave no response to addition of insulin. On the other hand giving choline chloride to the deficient animals elevated their sensitivity to insulin as shown in table 9. This result agrees well with Rodbell's experiment (14). The effect of insulin on cell membranes is not known. However, it can be postulated from our results that phospholipid in the cell plays a role in glucose transport across cell membranes. Thus, the rearrangement of cell structures would be caused by the injection of choline chloride into deficient animals, as a consequence of synthesis of lecithin or other choline-lipids. Presumably this rearrangement would be accompanied by the recon-

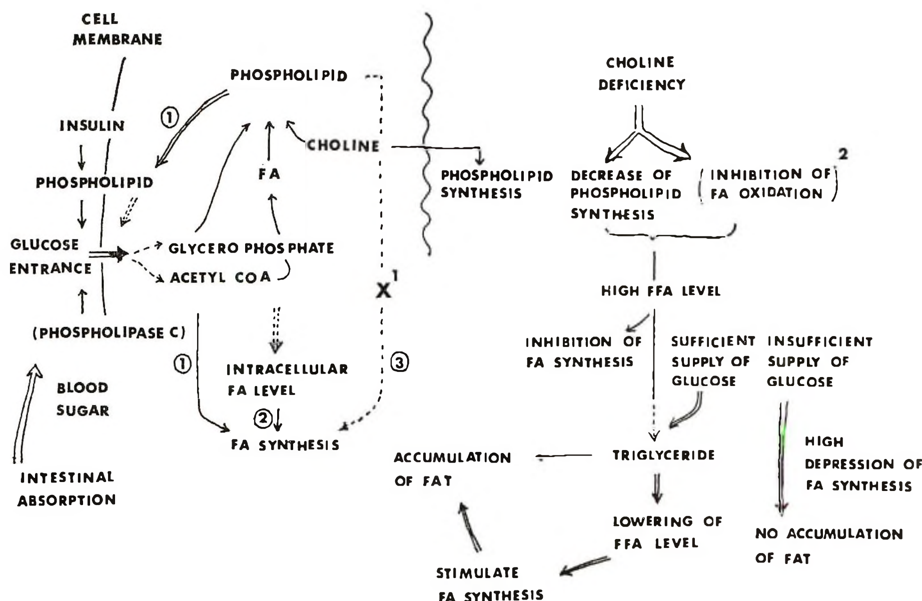

Fig. 1 Possible mechanisms of depression in fatty acid synthesis; X[1]: Possibly this represents a disturbance in the enzymatic system in fatty acid synthesis or changes of reaction field, although concrete evidence is not presented here;[2] this has been reported for rats (18, 19).

struction of the structural protein of the cells. Since injections of choline caused a marked enlargement of the liver as shown in tables 4, 6 and 7, the decrease of ^{14}C-phospholipid per unit protein at 20 hours after the injection, when it was compared with that at 6 hours, shown in table 5, may depend upon the enlargement of the liver. Therefore, this reconstruction of cell membrane certainly would be accompanied by the secondary increase of protein synthesis. However, it would be induced secondarily. And the marked enlargement of the liver, following the administration of choline, is similar in some points to the regenerated liver and the clarification of its mechanism remains to be resolved.

From those observations a possible mechanism of depressed FA synthesis, induced by feeding a choline-deficient diet, was summarized in figure 1. Feeding a choline-deficient diet induced a disturbance of intestinal absorption and the animals tended to become hypoglycemic. Also, permeability across the cell membranes was inhibited; consequently, utilization of glucose was suppressed significantly. The sensitivity of tissue to insulin disappeared with feeding the choline-deficient diet. These disturbances resulted in a decrease of liver glycogen and carcass lipid. Deficiency of intracellular choline inhibits phospholipid synthesis and fatty acid is not utilized for the synthesis. If the glucose supply is sufficient, FFA would be accumulated as triglyceride. However, if the glucose supply is insufficient the accumulation of FFA would increase and the increased FFA would inhibit fatty acid synthesis. Administration of choline to the deficient animals induced fatty acid synthesis through increasing phospholipid synthesis and decreasing the FFA level. Furthermore, the increase of glucose supply would be accompanied by increase of NADPH synthesis, which is needed for fatty acid synthesis.

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Alleviation of the Leg Abnormality in Zinc-deficient Chicks by Histamine and by Various Anti-arthritic Agents [1]

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ABSTRACT Two experiments were conducted with zinc-deficient chicks fed a diet based on soybean protein (13 ppm Zn) to assess the effects, on the "arthritis-like" or "perosis-like" leg defect, of feeding a number of commonly used anti-arthritic agents. Prevention of the leg deformity in chicks was compared with that elicited by dietary histamine dihydrochloride (0.2%) and by dietary zinc (88 ppm Zn). Either histamine or zinc essentially prevented the occurrence of leg abnormalities. Of the five anti-arthritic agents tested, four were highly effective in preventing the leg disorder. The effective agents and approximate dietary level (in percent) required for protection were: aspirin, 0.5 to 1.0; phenylbutazone, 0.2; cortisone acetate, 0.1; and indomethacin, 0.025. Dietary gold sodium thiosulfate (0.2%), the fifth anti-arthritic agent, had little effect, but it was not tested by intramuscular injection, the way it is usually administered to human patients. Histamine, as well as the anti-arthritic agents, had little or no effect on the symptoms of zinc deficiency other than the leg defect. Three other drugs related to histamine (pyrilamine maleate, an anti-histaminic, 0.2%; β-histine, a vasodilator, 0.3%; and aminoguanidine, a histaminase inhibitor, 0.1%) had no significant effect on the zinc-deficient chick. The possible use of the zinc-deficient chick in the study of arthritis and anti-arthritic agents is discussed.

O'Dell and his associates (1) reported that one of the defects caused by zinc deficiency in chicks was a leg abnormality. This defect was characterized by a stiff and unsteady gait, shortening and thickening of the long bones, swelling of the hocks, apparent failure of cartilage cell development in the epiphyseal plate region of the long bone and decreased osteoblastic activity in the bony collar. The results of Young and co-workers (2) and later work in our laboratory (3) confirmed the observation that chicks fed a low zinc diet based on isolated soybean protein developed the leg defects described by O'Dell et al. (1). However, chicks fed low zinc diets based on casein hydrolysate or egg white, although having the other signs of zinc deficiency, did not develop the leg abnormality (3).

Further studies (4, 5) on zinc-deficient chicks fed diets based on soybean protein demonstrated that large amounts (1–2% of the diet) of supplemental histidine, or smaller amounts (0.1–0.2% of the diet) of histamine, prevented the development of leg abnormalities but did not otherwise alleviate the zinc deficiency. A number of other histidine or histamine metabolites and related compounds were ineffective in alleviating the leg abnormality (5). The leg defect in chicks fed low zinc diets based on soybean protein was designated as "arthritis-like" or "perosis-like" on the basis of the outward signs (5). The stiff gait and the reluctance, but not complete disability, in movement suggested a painful joint. Furthermore, the joint was swollen and a twisting deformity was sometimes present, but "slipped tendon" was extremely rare. The observation that hydralazine can cause lesions in man resembling rheumatoid arthritis (6) and that this same agent causes a perosis in chicks (7) provides further evidence for a possible relationship between perosis and arthritis. Although the observations that have been made on the histopathology of the lesions of the bones and joints in zinc-deficient chicks (1, 2) provide no evidence for a

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close parallel to rheumatoid arthritis, it was speculated that the pathogenesis of the hock disorder in zinc-deficient chicks may have some features in common with that of certain arthritic conditions in humans. It was reasoned further that substances which act as anti-arthritic agents in man may also act as alleviators of the "arthritis-like" leg disorder in zinc-deficient chicks fed diets based on isolated soybean protein.

The present paper describes the effects of several types of anti-arthritic drugs (i.e., cortisone, aspirin, indomethacin, phenylbutazone, and gold thiosulfate) on the zinc-deficient chick. Because histamine effectively alleviated the "arthritis-like" leg disorder in zinc-deficient chicks (5), the 3 drugs pyrilamine maleate (an anti-histaminic), β-histine (a vasodilator like histamine), and aminoguanidine, a histaminase inhibitor), were also tested.

MATERIALS AND METHODS

Two experiments were conducted with one-day-old New Hampshire × Single Comb White Leghorn chicks without segregation according to sex. The birds were distributed at random into groups of 10 each and placed in a stainless steel battery at 37° to 40°. Feed and distilled water were provided ad libitum in aluminum troughs. Feed was mixed every 2 weeks and stored in a refrigerator until it was fed. The composition of the diet, based on soybean protein, was that described previously (3) except that 4 mg of folic acid/kg of diet were fed instead of 0.4 mg. In addition, the zinc-deficient diet used in the present study contained 5 ppm of supplemental zinc and analyzed 13 ppm total zinc on an air-dried basis (determined by a Perkin Elmer Model 214 atomic absorption spectrophotometer (3)). The 5 ppm of supplemental zinc were added to the diet to reduce mortality and to allow sufficient growth so that leg abnormalities were more prominent.

As controls in each experiment, a group of chicks were fed the zinc-deficient diet (severe leg deformity) and another group was fed the diet containing 80 ppm supplemental zinc (essentially free of leg deformities). Zinc was added to the diet in the form of zinc oxide.

In experiment 1, test groups of chicks fed the zinc-deficient diet were fed the following compounds [2] (in percent of the air-dried diet), respectively: histamine dihydrochloride, 0.2; phenylbutazone, 0.1; indomethacin, 0.1; cortisone acetate, 0.05; aspirin (acetylsalicylic acid), 0.2; pyrilamine maleate, 0.2; β-histine, 0.3; and aminoguanidine bicarbonate, 0.2.

In experiment 2, the compounds tested as additions to the zinc-deficient diet were (in percent of the air-dried diet), respectively: histamine dihydrochloride, 0.2; phenylbutazone, 0.2; indomethacin, 0.1, 0.05, and 0.025; aspirin (acetylsalicylic acid), 1.0 and 0.5; cortisone acetate, 0.1; and gold sodium thiosulfate,[3] 0.2.

During the course of each experiment, the chicks were weighed weekly and observed for abnormalities. When the chicks were 4 weeks of age, body weights, leg scores and feather scores were taken. Legs were scored using a scale of 1 through 5. The following designations were given: 1, indicates a chick with normal legs; 2, a chick with legs slightly shortened and thickened, and walking with toes pointed outward more than normal; 3, a chick with legs which were moderately shortened and thickened and with moderately swollen hocks, such that walking ability was hampered slightly; 4, a chick with shortened and thickened legs with swollen hocks causing a very stiff and unsteady gait; and 5, a chick with extremely shortened and thickened legs with hocks so twisted and swollen that the chick could hardly walk. Feathers were scored using a scale of 1 through 5. The following designations were given: 1, wing feathers were normal; 2, wing feathers were slightly ragged, but not typical of zinc-deficient chicks (4); 3, the inner portion

[2] The sources of the compounds tested were as follows: Histamine dihydrochloride, Nutritional Biochemicals Corporation, Cleveland; phenylbutazone (Butazolidin), Geigy Pharmaceuticals, Ardsley, New York; indomethacin (Indocid), Merck and Company, Inc., Rahway, New Jersey; cortisone acetate, Mann Research Laboratories, Inc., New York; aspirin (acetylsalicylic acid), Sigma Chemical Company, St. Louis; pyrilamine maleate, K and K Laboratories, Inc., Plainview, New York; β-histine (Serc), Unimed, Inc., Morristown, New Jersey; and aminoguanidine bicarbonate, Aldrich Chemical Company, Inc., Milwaukee. The authors acknowledge with thanks the following companies for supplying drugs gratis: Geigy Pharmaceuticals, Merck and Company, Inc., and Unimed, Inc.

[3] Prepared from gold chloride and sodium thiosulfate as described in the Merck Index, ed. 7, 1960 Merck and Company, Inc., Rahway, New Jersey, p. 495.

(secondaries) of the feathers showed some barbules or barbicels which were not interlocked properly (less than 25%), which caused the inner part of the feathers to have a slightly ragged appearance while the outer part of the feather appeared to be normal; 4, same as 3 except for a much greater proportion (more than 25%) of poorly functioning barbules or barbicels; and 5, very abnormal wing feathers with brittle and broken rachises and with the entire inner portions showing no properly interlocked barbules or barbicels. The rating systems were reproducible, as approximately 80% of the time two independent observers arrived at the same value. In the other 20% of the cases, the observers did not differ by more than one value. After the weights and scores were taken, blood was withdrawn from the chick by heart puncture with a heparinized needle. The chick was decapitated, and the legs were removed and stored at − 8° for later analysis. Length and width measurements of the femurs were determined by removing the flesh from the bone by rubbing with cheesecloth and measuring the smallest diameter and the largest length with micrometers. In experiment 2, the gizzards were also inspected to screen for ulcerogenic activity of any of the drugs. The gizzards were scored using a scale of 1 through 5. The following designations were given: 1, normal; 2, slight erosions, or 1 erosion; 3, 2 to 3 erosions; 4, more than 3 erosions; and 5, ulcers.

Statistical analysis was by Duncan's multiple range test for unequal replication (8).

RESULTS

The results of experiment 1 are shown in table 1. As reported previously (5), histamine alleviated the leg abnormality in the zinc-deficient chick. Histamine significantly decreased the leg score and significantly increased the length-to-width ratio of the femur. However, histamine-treated chicks were still severely zinc-deficient as the final body weight was not significantly different from that of the zinc-deficient controls; also, addition of zinc to the histamine-supplemented diet produced a marked increase in growth rate.

Cortisone acetate (0.05% of the diet) and indomethacin (0.1% of the diet) had effects similar to the effect of histamine. Both of these anti-arthritic and anti-inflammatory drugs alleviated the "arthritis-like" syndrome in the zinc-deficient chick, as indicated by the significantly decreased leg scores and, in the case of indomethacin, by the significantly increased length-to-width ratio of the femurs. Neither drug had an effect on body weight. At the levels tested, the other two anti-arthritic agents, aspirin and phenylbutazone, gave indications of alleviating leg abnormalities

TABLE 1

Body weights, leg scores, feather scores and femur length-to-width ratios at 4 weeks of age in chicks fed diets based on soybean protein and supplemented with various anti-arthritic and related compounds (exp. 1)

Treatment	Zinc-supplemented	Body wt	Mortality	Leg score [1]	Feather score [1]	Length/width ratio of femur
% of diet	*ppm*	*g*	*%*			
None	5 [2]	178 [ab 3]	0	4.4 [a]	4.4 [a]	9.6 [ab]
None	80	361 [d]	20	1.2 [d]	1.4 [d]	11.8 [c]
0.2% histamine dihydrochloride	5	153 [a]	0	2.0 [cd]	2.7 [c]	11.4 [c]
0.2% histamine dihydrochloride	80	246 [c]	0	1.3 [d]	1.6 [c]	12.3 [c]
0.1% phenylbutazone	5	187 [ab]	0	3.8 [ab]	4.0 [c]	9.5 [ab]
0.1% indomethacin	5	141 [a]	70	1.7 [cd]	5.0 [a]	12.6 [cd]
0.05% cortisone acetate	5	177 [ab]	10	2.9 [bc]	3.2 [bc]	10.1 [b]
0.2% aspirin	5	199 [b]	10	3.8 [ab]	4.0 [ab]	9.3 [ab]
0.2% pyrilamine maleate	5	158 [a]	20	3.4 [ab]	3.4 [ab]	10.2 [b]
0.3% β-histine	5	156 [a]	0	3.8 [ab]	4.3 [a]	9.4 [ab]
0.1% aminoguandine bicarbonate	5	184 [ab]	20	4.1 [a]	3.9 [ab]	8.8 [a]

[1] 1 = normal; 5 = very abnormal, see text.
[2] Diet contained a total of 13 ppm zinc by analysis.
[3] Values within a column followed by the same letter are not significantly different ($P > 0.05$) from each other.

in the zinc-deficient chick, but the effects were statistically nonsignificant by the criteria used.

Pyrilamine maleate, β-histine and aminoguanidine bicarbonate, at the levels tested, had no significant effect on the zinc-deficient chick.

Although indomethacin alleviated the leg abnormality in zinc-deficient chicks, it had a detrimental side effect. Seven of 10 chicks started on experiment died before the experiment was terminated. Upon postmortem examination of four of these chicks, it was found that the gizzards were ulcerated severely and had perforated, causing the abdominal cavities to be filled with fluid.

From experiment 1, it appeared that indomethacin was being fed at an excessive level. Furthermore, since cortisone acetate was not as effective as histamine in overcoming leg abnormalities, and because phenylbutazone and aspirin had only slight, nonsignificant effects on the zinc-deficient chick, it was concluded that effective levels of these compounds may not have been fed in experiment 1. The second experiment was then conducted with drug levels adjusted accordingly.

The data for experiment 2 are shown in table 2. All anti-arthritic agents at the levels tested in this experiment (except for the borderline improvement as the result of gold sodium thiosulfate treatment) gave results which were not significantly different from that of histamine treatment. The improvement in legs was so marked that none of the groups, except the one fed gold sodium thiosulfate, had length-to-width ratios of femurs significantly different from those of chicks fed the zinc-supplemented control diet. The chicks fed indomethacin, cortisone or aspirin had leg scores which were not significantly different from those of zinc-supplemented controls. The chicks appeared still to be zinc-deficient, as they showed depressed growth, and with the possible exception of the surviving chicks fed 0.1% indomethacin, had abnormal feathering (high feather scores).

Histamine, phenylbutazone, aspirin, and indomethacin had detrimental effects on the gizzards as indicated by the increased gizzard scores. Indomethacin appeared to cause the greatest amount of gizzard ulceration; some of the gizzards were ulcerated so severely that perforations were present. When indomethacin was fed at 0.1% of the diet, 80% mortality resulted. At 0.05% of the diet, indomethacin caused death of 70% of the chicks. At 0.025% indomethacin, the mortality rate was decreased to 10%, but the gizzards of the

TABLE 2

Body weights, leg scores, feather scores, gizzard scores and femur length-to-width ratios at 4 weeks of age in chicks fed diets based on soybean protein and supplemented with various anti-arthritic agents (exp. 2)

Treatment	Zinc-supplemented	Body wt	Mortality	Leg score [1]	Feather score [1]	Gizzard score [1]	Length/width ratio of femur
% of diet	*ppm*	*g*	*%*				
None	5 [2]	148 [ab 3]	40	4.5 [a]	4.2 [a]	2.0 [a]	10.0 [a]
None	80	274 [c]	0	1.2 [e]	1.3 [d]	2.3 [ab]	12.3 [b]
0.2% histamine dihydrochloride	5	132 [ab]	0	2.1 [cd]	2.8 [bc]	4.5 [c]	11.1 [ab]
0.2% phenylbutazone	5	131 [ab]	30	2.3 [c]	4.1 [a]	3.9 [c]	11.1 [ab]
0.1% indomethacin	5	130 [ab]	80	1.5 [cde]	1.5 [cd]	5.0 [c]	12.4 [b]
0.05% indomethacin	5	104 [a]	70	1.3 [cde]	4.0 [ab]	5.0 [c]	12.6 [b]
0.025% indomethacin	5	130 [ab]	10	1.7 [cde]	3.6 [ab]	4.6 [c]	12.0 [ab]
1.0% aspirin	5	107 [a]	0	1.3 [de]	3.0 [abc]	3.9 [c]	12.4 [b]
0.5% aspirin	5	132 [ab]	10	2.2 [c]	4.1 [a]	3.6 [c]	11.6 [ab]
0.1% cortisone acetate	5	127 [ab]	10	1.8 [cde]	3.3 [ab]	3.3 [bc]	11.3 [ab]
0.2% gold sodium thiosulfate	5	155 [b]	20	3.7 [b]	4.0 [ab]	2.1 [a]	10.2 [a]

[1] 1 = normal; 5 = very abnormal, see text.
[2] Diet contained a total of 13 ppm zinc by analysis.
[3] Values within a column followed by the same letter are not significantly different ($P > 0.05$) from each other.

chicks usually showed severe ulceration which resulted in the high gizzard score of 4.6.

Gold sodium thiosulfate did not have a marked effect on the leg abnormality, but it improved slightly, but significantly, the leg score. Further studies are needed to ascertain whether gold sodium thiosulfate administered by intramuscular injection as it is for human arthritic patients, or at a higher dietary level, will cause a definite alleviation of the "arthritis-like" leg disorder.

DISCUSSION

The apparently paradoxical result that histamine, which is supposedly a mediator of the early phases of inflammation (9), and the several anti-inflammatory compounds had a similar alleviating effect on the "arthritis-like" leg deformity of zinc-deficient chicks is difficult to explain at our present state of knowledge. However, histamine has also been shown to moderate the lesions of "formalin-induced" arthritis of rats (10, 11).

Because drugs used in the treatment of arthritic syndromes, such as rheumatoid arthritis and osteoarthritis, in man, had such a dramatic effect on the leg abnormality of zinc-deficient chicks fed a diet based upon isolated soybean protein, one may postulate that the pathogenesis of the leg abnormality may be similar, in at least certain respects, to some arthritic syndromes in man. If this similarity exists, it is possible that the "arthritis-like" leg abnormality of chicks can play a role in two investigational fields, (a) elucidating the biochemical causes and suggesting methods of prevention of arthritic syndromes in man, and (b) the screening of compounds for possible anti-arthritic activity and study of their mechanisms of action.

The importance of (a) above needs no explanation, for the exact etiology, pathogenesis, and lasting cures for arthritic conditions such as rheumatoid arthritis and osteoarthritis are not known. This may, in part, be due to the fact that no species of experimental animal is known to have a natural arthritis similar to that of rheumatoid arthritis or osteoarthritis in man. Many "arthritis-like" syndromes have been produced in animals but most are not very satisfactory for the study of rheumatoid arthritis or osteoarthritis. Gardner (12) has reviewed many of these syndromes and has given reasons why they are unsatisfactory for the study of arthritic syndromes like rheumatoid arthritis. Most of the experimental arthritic syndromes are created by the injection of exogenous substances, for example, urate crystals in dogs (13), "adjuvant" in rats (14), or bacteria in swine (15).

In this investigation, a leg abnormality which responds to anti-arthritic agents was created in chicks without the injection of an exogenous substance, but simply by making alterations in the diet. The abnormality can be obtained by feeding soybean protein, but not casein hydrolysate or dried egg white, as the amino acid source for zinc-deficient chicks (3). However, it has been observed in our laboratory [4] that this leg abnormality can also be created in zinc-deficient chicks fed the appropriate amino acid diets (apparently, low levels of both histidine and zinc are needed); therefore, this effect does not specifically depend on feeding isolated soybean protein, but relates to the amino acid pattern of the diet. Possibly some disturbance in the metabolism of zinc or of histidine or histamine may be important in the etiology of rheumatoid arthritis or osteoarthritis in man. Of possible significance in this respect is the observation that adult human patients with rheumatoid arthritis have higher-than-normal concentrations of zinc in the synovial fluid and excrete about threefold more zinc in the urine than controls.[5] Moreover, a depressed concentration of blood serum histidine has been suggested as a diagnostic aid for rheumatoid arthritis.[6] Even if the overt causes of the arthritic syndromes in man and those produced in chicks are different, as appears likely, the basic biochemical

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[4] Nielsen, F. H. 1967 Relationship of histidine, its metabolites and other factors to the "arthritic" syndrome in zinc-deficient chicks. Ph.D. Thesis, University of Wisconsin.

[5] Bonebrake, R. A., J. T. McCall, G. G. Hunder and H. F. Polley 1967 Zinc accumulation in synovial fluid. Federation Proc., *26:* 523 (abstract).

[6] Gerber, D. A., and M. G. Gerber 1967 Decreased concentration of histidine in the serum of patients with rheumatoid arthritis — a new diagnostic aid. Arthritis Rheum., *10:* 279 (abstract).

lesion may be the same and studies of this lesion should be pursued in zinc-deficient chicks.

The possibility of "rheumatoid-like" arthritis occuring in chicks is not remote. It has been shown that chronic administration of hydralazine, used for the treatment of hypertension in humans, can cause lesions resembling rheumatoid arthritis in man (6). Feeding hydralazine to chicks results in a perosis, which is similar in many respects to the hock disorder studied here, and which has reportedly been overcome by manganese supplementation (7). However, manganese supplementation does not affect the leg disorder caused by zinc deficiency (3) and histamine is *not* effective in overcoming the perosis caused by manganese deficiency.[7] Clearly, the perosis caused by manganese deficiency differs in important respects from that caused by zinc deficiency.

Use of the leg defect in zinc-deficient chicks for screening possible anti-arthritic, an anti-inflammatory, compounds and studying their mechanism of action is suggested. Many screening tests now used are not closely related to rheumatoid arthritis or osteoarthritis. For example, the Evans Blue pleural effusion test measures the amount of reduction of the exudative response to the intrapleural injection of Evans Blue (16). In rats with "adjuvant"-induced arthritis, several known anti-inflammatory, or anti-arthritic, drugs have been shown to decrease the foot volume and "inflammation unit" measurements (17). However, as in the case of the Evans Blue pleural effusion test, these measurements are indexes only of a general inflammatory response to the injection of an exogenous substance. With the zinc-deficient chick fed a diet based on isolated soybean protein, possible anti-arthritic compounds can be tested against an "arthritis-like" syndrome not caused by injection of exogenous substances. The gastrointestinal ulceration produced by certain drugs can also be determined simultaneously.

That an "arthritis-like" syndrome which responds to anti-inflammatory agents can result in chicks from a nutritional insult, may have further implications for the cause of arthritis in man.

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An Unrecognized Nutrient for the Guinea Pig [1,2]

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ABSTRACT An attempt was made to identify or characterize the components of natural products which stimulate the growth rate of guinea pigs fed purified diets. Animals were fed for 4 weeks a soybean protein basal diet that contained 0.5% added methionine, 15% woodpulp and 200 mg of ascorbic acid/100 g. The growth rate was increased when this diet was supplemented with either raw cabbage or alfalfa. Similar results were obtained when a casein basal diet was fed. The growth-promoting component of cabbage was identified as ascorbic acid. When ascorbic acid was supplied as single oral doses of up to 60 mg/day it had no effect, but the consumption of a continuously available ascorbic acid solution (2 mg/ml) significantly increased the growth rate. It was concluded that cabbage stimulated growth by virtue of providing a more nearly continuous supply of ascorbic acid. Alfalfa contains a growth stimulant other than ascorbic acid. When the alfalfa was oven-dried it did not supply a significant amount of ascorbic acid, but supported an excellent growth rate. Water and ethanol extraction did not remove the active component and the activity of the residue could not be attributed to its ash or cellulose content. Alfalfa supplementation increased the ascorbic acid content of adrenal and spleen tissues slightly, but not to the same extent as ad libitum consumption of cabbage and ascorbic acid solution.

Since the isolation of vitamin B_{12}, numerous claims have been made for the existence of unidentified nutrients in natural products. A large proportion of these investigations have used the chick as the experimental subject, but there are reports that the guinea pig requires unrecognized compounds to support the maximal growth rate.

Ershoff (1) reported a growth response to desiccated alfalfa when guinea pigs were fed a mineralized dried milk ration, but not when they were fed a semisynthetic diet. However, Reid and Mickelsen (2) obtained a significant increase in growth rate by supplementation of a casein-base diet with 27% of alfalfa meal so as to maintain a constant level of nitrogen. More recently, Lakhanpal et al. (3) have also presented evidence for an unidentified growth factor in alfalfa and other plant sources. Certain plant products have been shown to decrease susceptibility of guinea pigs to radiation injury (4) and salmonellosis (5).

The guinea pig is nutritionally unique among lower animals in that it requires ascorbic acid. A vitamin C-sparing factor, which elevates tissue levels of ascorbic acid, has been postulated (6, 7). Bioflavonoids have been shown to increase tissue ascorbic acid levels when fed as supplements to an ascorbic acid-containing diet, but they did not retard the development of scurvy with a scorbutogenic diet (8, 9). Crampton and Lloyd (10) concluded that rutin improves the biological potency of ascorbic acid when ascorbic acid is fed at sub-minimal levels.

In a preliminary report [4] it was shown that raw cabbage stimulates the growth rate of guinea pigs and protects them against salmonellosis. The growth-stimulating component was destroyed by drying but the salmonellosis resistance factor was stable (5). The present paper is concerned with the nature of the labile component in cabbage and presents evidence for an unrecognized nutrient in alfalfa.

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EXPERIMENTAL

Weanling guinea pigs, 3 to 8 days of age, produced in our colony and weighing about 125 g, were housed on raised wire floors in groups of four. Because of other demands for the males, for the most part, females were used in this study. Control animals were started at approximately monthly intervals so that at least one group was fed the basal diet concurrently with those fed the respective supplements. Since the growth rates of the basal groups did not vary significantly, those fed the test diets were compared with the total number fed the basal diet, with the t test used as a measure of statistical significance.

The composition of the basal diets is given in table 1. Supplements were added at the expense of the total diet. Ascorbic acid was included in the basal diets, but in some of the later experiments it was omitted from the diet and supplied as a daily oral dose. Food and water were supplied fresh once daily and were available ad libitum. The food was moistened with water to form a thick paste just before it was offered to the animals. Although this practice resulted in loss of ascorbic acid, assays showed that sufficient remained to meet the recommended allowance.

TABLE 1

Composition of basal diets

	Casein basal	Soy protein basal
	%	%
Washed casein [1]	30.0	—
Isolated soy protein [2]	—	30.0
Sucrose	43.3	42.1
Cellulose [3]	15.0	15.0
Soybean oil	4.0	4.0
Salts I [4]	4.0	—
Salts II [5]	—	5.0
Potassium acetate	2.7	2.5
Magnesium oxide	0.5	0.5
Ascorbic acid	0.2	0.2
Choline chloride	0.1	0.2
DL-Methionine	0.2	0.5
Chlortetracycline·HCl	0.0025	0.0025
Water-soluble vitamins [6]	+	+
Fat-soluble vitamins [7]	+	+

[1] Nabb and O'Dell (5).

[2] Promine R, Central Soya Company, Inc., Chicago.

[3] Wood pulp (Solka Floc), Brown Company, Berlin, New Hampshire.

[4] Salts I supplied: (g/100 g of diet) $CaCO_3$, 2.05; $MgCO_3$, 0.10; $MgSO_4$, 0.05; $MnSO_4 \cdot H_2O$, 0.08; NaCl, 0.28; KCl, 0.45; KH_2PO_4, 0.83; $FePO_4$ (soluble), 0.16; $CuSO_4 \cdot 5H_2O$, 0.0056; $ZnSO_4 \cdot 7H_2O$, 0.0025; $CoCl_2 \cdot 6H_2O$, 0.003; $AlK(SO_4)_2 \cdot 12H_2O$, 0.0007; NaF, 0.004; and KI, 0.003.

[5] Salts II supplied: (g/100 g of diet) $CaCO_3$, 0.90; $CaHPO_4$, 2.04; $MgSO_4$, 0.30; $MnSO_4 \cdot H_2O$, 0.06; NaCl, 0.26; Na_2HPO_4, 0.64; KCl, 0.77; Fe citrate, 0.036; $CuSO_4$, 0.002; $ZnCO_3$, 0.0143; and KIO_3, 0.0017.

[6] Water-soluble vitamins supplied: (mg/100 g diet) thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; niacin, 5.0; folic acid, 0.6; inositol, 100; D-biotin, 0.02; and cyanocobalamin, 0.003.

[7] Fat-soluble vitamins supplied per 100 g diet: vitamin A palmitate, 2000 IU; vitamin D_3, 285 IU; α-tocopheryl acetate, 2.0 mg; menadione, 1.0 mg; ethoxyquin (Santoquin, Monsanto Company, St. Louis), 12.5 mg.

Analytical methods Ascorbic acid in fresh cabbage, cabbage juice, dehydrated alfalfa meal and the basal rations was determined by indophenol titration (11). The 2, 4-dinitrophenylhydrazine procedure of Roe and Kuether (12) was used to determine ascorbic acid in adrenal, spleen and liver. The methods reported by the vitamin E panel of the SAC (13) and Booth and Bradford (14) were modified for the determination of the lipid-soluble reducing substances in alfalfa meal.

Cabbage fractions The outside leaves of cabbage were removed and the remainder was divided into portions of approximately 300 g. These were supplied either ad libitum or on a dry-matter basis as indicated. Cabbage was dried in a forced-draft oven at 60° after being chopped to near salad consistency.

Cabbage juice was prepared by grinding the raw cabbage in a food chopper and straining through cheesecloth. The residue which remained was labeled cabbage pulp. The combination of juice and pulp was labeled blended cabbage.

When autoclaved, the cabbages were cut into eighths, wrapped in cheesecloth and heated at 115° for 20 minutes. The cooking juice was decanted and the juice expressed from the wrapped heads in a manual press. The pulp remaining was dried in a forced-draft oven and ground in a hammer mill. When the juice was incorporated into the diet it was concentrated in vacuo to a syrup.

Alfalfa fractions Since commercial alfalfa meal did not consistently give a growth response, alfalfa meal was prepared from fourth-cutting alfalfa grown on the Experiment Station farm and harvested in late September or early October. The freshly cut plants were brought to

the laboratory and dried at 80° in a forced-draft oven. The dried material was ground in a hammer mill and stored under refrigeration until used. Such preparations retained growth-promoting activity for at least 3 years.

An aqueous extract was prepared as follows: Oven-dried alfalfa meal (1500 g) was placed in 20 liters of water and heated to boiling with live steam. The suspension was allowed to steep 30 minutes, and was then drained through nylon net. The residue was re-extracted until the filtrate was essentially colorless; usually 6 times sufficed. The final residue was dried in a forced-draft oven. It constituted about 66% of the original weight. The water extract was concentrated to a syrup (20–40% dry matter) in a vacuum evaporator. The dry matter yield was about 30% of the original alfalfa meal.

An ethanol extract was prepared as follows: Oven-dried alfalfa meal (2.5 kg) was refluxed with 95% ethanol (10:1) for 4 hours and after cooling the supernatant solution was decanted. The residue was re-extracted a total of 10 times. After 6 extractions the extract was nearly colorless, but additional extractions were made to assure removal of all ethanol soluble materials. The yield of residue was about 80% of the starting material. The ethanol extracts were combined and concentrated in a vacuum evaporator to a syrup-like consistency. The dry-matter yield was about 15% of the original starting material.

RESULTS

The growth responses obtained when the basal diets were supplemented with cabbage and various cabbage fractions are shown in table 2. Raw cabbage significantly stimulated the growth rate of female guinea pigs fed the casein basal diet when supplied at dry-matter levels equivalent to 8.5% of total food consumption or ad libitum (approximately 25%

TABLE 2

Effect of cabbage and cabbage fractions on the growth of female guinea pigs

Supplement	Amount [1]	No. animals	Daily gain (4-wk)
	%		g
Casein basal			
None	—	36	5.5 ± 0.11 [2]
Raw cabbage	8.5	8	6.7 ± 0.47 ** [4]
Raw cabbage	ad lib.	8	7.3 ± 0.35 **
Oven-dried cabbage	15.0	11	5.6 ± 0.34
Blended cabbage mixed in diet	15.0	16	6.1 ± 0.22
Blended cabbage fed separately	15.0	4	7.2 ± 0.43 **
Fresh cabbage juice, 3 × daily in bottle	ad lib.	4	5.8 ± 0.41
Fresh cabbage pulp, 3 × daily, separately	ad lib.	4	6.8 ± 0.44 *
Raw cauliflower	ad lib.	11	7.2 ± 0.26 **
Raw endive	ad lib.	8	7.2 ± 0.33 **
Soybean protein basal			
None	—	38	6.2 ± 0.18
Raw cabbage	ad lib.	12	7.6 ± 0.24 **
Autoclaved cabbage pulp in diet	5.0	8	6.1 ± 0.25
Autoclaved cabbage juice in bottle [3]	ad lib.	28	7.4 ± 0.19 **
Autoclaved cabbage juice conc in diet	20.0	8	5.9 ± 0.39
Ascorbic acid 30 mg per os, daily	—	24	6.2 ± 0.18
Ascorbic acid 60 mg per os, daily	—	24	6.5 ± 0.11
Ascorbic acid in bottle, 2 mg/ml	ad lib.	16	7.1 ± 0.31 **
α-Tocopheryl acetate	0.01	24	7.0 ± 0.21 **
α-Tocopheryl acetate + ascorbic acid soln. (2 mg/ml, ad libitum)	0.01	16	7.0 ± 0.25 **
Rutin mixed in diet	0.1	12	6.6 ± 0.22
Rutin 30 mg per os, daily	—	8	6.4 ± 0.19

[1] Percentages on dry-matter basis.
[2] SE of mean.
[3] Autoclaved juice prepared fresh daily and fed in bottle.
[4] Different from basal by Student's t test, * $P < 0.05$, ** $P < 0.01$.

of total). The growth-promoting activity was destroyed by oven-drying. When blended fresh cabbage was mixed with the basal diet at a level of 15.0% of the dry matter, there was not a statistically significant improvement in growth rate. However, when the blended cabbage was fed separately from the basal diet, the results were equivalent to those obtained with unchopped portions. Juice prepared fresh 3 times daily and offered ad libitum in a water bottle was inactive. The pulp remaining after extraction of the juice retained some activity. Thus, it appears that the active component was destroyed when the cells were broken, probably being catalyzed by an enzyme as well as by a dietary constituent. Another member of the brassica family, cauliflower, and endive stimulated growth to the same extent as cabbage.

The activity of cabbage fractions was also determined using the soybean protein basal diet. In agreement with the results of Reid and Mickelsen (2), the growth rate with the unsupplemented soybean protein basal was greater than that with the casein basal diet. Even though this diet had a more adequate amino acid balance for the guinea pig, the raw cabbage supplement elicited a growth response of 1.4 g per day. In an attempt to eliminate possible enzymatic destruction of the growth-promoting factor, the cabbage was autoclaved before blending to prepare the juice. The pulp, when dried and mixed in the diet, was inactive. In contrast with fresh juice, the autoclaved cabbage juice, supplied ad libitum, stimulated growth to nearly the same extent as fresh raw cabbage. A concentrate of the autoclaved juice mixed in the diet produced no stimulation in the growth rate. Thus, the growth stimulant in cabbage was water-soluble and readily destroyed in solution when catalzyed by undenatured cell components or by contact with the diet.

The labile properties of the active component of cabbage resemble those of ascorbic acid. Whereas the ascorbic acid of the fresh juice decreased to 30% of the initial value within 60 minutes, there was only a slight decrease in the ascorbic acid content of autoclaved cabbage juice over the same period. The ascorbic acid content of the dry basal diet was 200 mg per 100 g. When moistened, the level of reduced ascorbic acid decreased to approximately 100 mg per 100 g in a 6-hour period. Although no gross signs of scurvy were detected, the effect of supplemental ascorbic acid was determined and the results are shown in table 2. Daily oral supplements of 30 or 60 mg of additional ascorbic acid did not increase the growth rate. However, if the ascorbic acid was provided free choice as a solution in a water bottle, the growth rate was increased to 7.1 g per day, a highly significant increase over the rate attained when the basal diet was fed. During the first week the young animals were given the ascorbic acid solution as the only source of water. During the remainder of the experimental period, both fresh water and the ascorbic acid solution were available. Consumption of ascorbic acid during this free-choice period ranged from 200 to 300 mg per animal per day. In view of the properties of the cabbage factor and the fact that a continuous supply of ascorbic acid solution stimulated growth, it is probable that the major effect of ad libitum raw cabbage is to provide a continuous source of ascorbic acid.

Other reducing substances were also tested for growth-promoting activity and some of the results are iucluded in table 2. The addition of α-tocopheryl acetate in the amount of 10 mg per 100 g of diet, increased the growth rate of females. A combination of α-tocopheryl acetate and ascorbic acid was no better than either alone. The bioflavonoid, rutin, mixed in the diet or given as a single oral dose, did not stimulate the growth rate. Two other compounds not shown in the table, ubiquinone at 1 mg per day and erythorbic acid at 30 mg per day, were inactive.

In view of the fact that the mode of ascorbic acid supplementation affects growth response, in subsequent experiments, the basal diet was prepared without ascorbic acid and the vitamin supplied as a daily oral dose of 30 mg per animal. Table 3 summarizes the results obtained under these conditions with supplements of alfalfa and alfalfa fractions. Alfalfa, freshly cut daily and offered ad libitum,

TABLE 3

Effect of alfalfa and alfalfa fractions upon the growth rate of weanling female guinea pigs

Supplement [1]	Amount [2]	No. animals	Daily gain (4-wk)
	%		g
None	—	24	6.2 ± 0.18 [3]
Fresh alfalfa daily	ad lib.	12	8.3 ± 0.28 ** [4]
Oven-dried alfalfa meal	2.5	16	6.9 ± 0.23 *
Oven-dried alfalfa meal	5.0	24	7.2 ± 0.20 **
Oven-dried alfalfa meal	10.0	19	6.7 ± 0.17 *
Water extract of oven-dried alfalfa	5.0	12	6.3 ± 0.30
Water-insoluble residue of oven-dried alfalfa	5.0	16	7.2 ± 0.20 **
Ethanol extract of oven-dried alfalfa	1.0	24	6.6 ± 0.26
Ethanol insoluble residue of oven-dried alfalfa	5.0	24	7.3 ± 0.20 **
Cellulose (wood pulp)	5.0	16	6.6 ± 0.16
Alfalfa ash	1.0	16	6.4 ± 0.18
Oven-dried alfalfa and ascorbic acid soln. (2 mg/ml, ad lib.)	5.0	20	6.8 ± 0.22 *
Oven-dried alfalfa and α-tocopheryl acetate (0.01%)	5.0	19	6.8 ± 0.14 *

[1] Soybean protein basal diet without added ascorbic acid. Each animal received 30 mg ascorbic acid per os daily unless other ascorbic acid supplemenation is indicated.
[2] Percentages on dry-matter basis.
[3] SE of mean.
[4] Difference from basal statistically significant, * $P < 0.05$; ** $P < 0.01$.

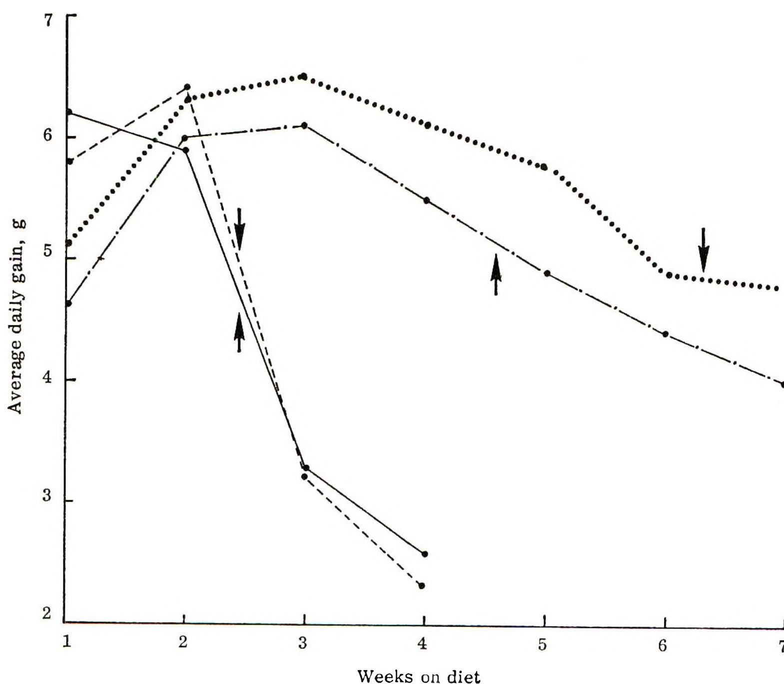

Fig. 1 Effect of oven-dried alfalfa on the response of guinea pigs fed a scorbutogenic diet or a suboptimal amount of ascorbic acid. ——— basal scorbutogenic diet; — — — — basal plus 5% oven-dried alfalfa, —·—·—· 0.5 mg of ascorbic acid daily per os, ... 0.5 mg of ascorbic acid daily per os plus 5% oven-dried alfalfa. Average time to appearance of gross signs of scurvy indicated by arrow.

gave a significant increase in the growth rate. In contrast with oven-dried cabbage, oven-dried alfalfa retained a significant portion of its activity. The 5% level gave the best rate of gain although the difference between 2.5 and 5% was not highly significant. It is probable that the activity lost during drying was due to

ascorbic acid and that another growth stimulant remained.

The oven-dried alfalfa meal, as assayed by the 2,4-dinitrophenylhydrazine procedure of Roe and Kuether (12), contained about 10 mg of ascorbic acid per 100 g. Thus, when added at the 5% level it provided less than 0.5 mg per 100 g of diet or a daily intake of approximately 0.1 mg, an insignificant amount compared with the 30-mg daily dose. Furthermore, it was determined biologically that there was insufficient ascorbic acid present in 5% of oven-dried alfalfa to retard the development of scurvy in guinea pigs fed a scorbutogenic diet (fig. 1).

As shown in table 3, the water extract of oven-dried alfalfa was inactive, but the insoluble residue was fully as active as the starting material. Similarly, the alcohol extract was inactive and the insoluble residue active. That the stimulation was not simply a function of bulk is shown by the failure of additional cellulose (total of 20%) to give a significant improvement in the growth rate. The alfalfa factor is not inorganic in nature as shown by the inactivity of alfalfa ash. Neither ad libitum ascorbic acid solution nor additional α-tocopheryl acetate when supplied in combination with oven-dried alfalfa meal supported a greater rate of gain than any single supplement. Furthermore, the growth-promoting effect of dehydrated alfalfa could not be due to its ascorbic acid content.

There might be an ascorbic acid-economizing factor in alfalfa which either reduces the need for exogenous ascorbic acid or reduces its rate of destruction in the body. In either case an increase in the tissue level should result. Consequently the ascorbic acid content of adrenal, spleen and liver was determined and the results are presented in table 4. Both fresh cabbage and ascorbic acid solution more than doubled the ascorbic acid content of the tissues analyzed. The oven-dried alfalfa supplement also increased the adrenal ascorbic acid level in males ($P < 0.05$), but did not have a statistically significant influence in females. Spleen and liver values were not affected by the supplement. Males fed the water and ethanol-insoluble residue had higher con-

TABLE 4

Effect of natural product supplements on tissue ascorbic acid in guinea pigs

Supplement [1]	Females				Males			
	Gain [2]	Adrenal	Spleen	Liver	Gain	Adrenal	Spleen	Liver
	g	*mg/100 g*	*mg/100 g*	*mg/100 g*	*g*	*mg/100 g*	*mg/100 g*	*mg/100 g*
None (30 mg ascorbic acid)	6.6(16)	68.7 ± 5 [3]	24.1 ± 2	7.9 ± 1	7.0(16)	66.8 ± 2	24.7 ± 1	11.2 ± 1
Ad lib. fresh cabbage	7.6(8)	187 ± 7 ** [4]	53.4 ± 3 **	30.1 ± 2 **	—	—	—	—
Ad lib. ascorbic acid soln. (2 mg/ml)	6.8(8)	169 ± 10 **	54.8 ± 1 **	40.1 ± 4 **	8.1(12)	153 ± 7 **	57.2 ± 3 **	38.8 ± 2 **
5% Oven-dried alfalfa meal	7.0(9)	73.4 ± 5	23.3 ± 2	7.5 ± 1	7.6(8)	78.6 ± 6 *	30.1 ± 2	11.0 ± 2
5% Water-insoluble residue of oven-dried meal	—	—	—	—	7.7(8)	73.3 ± 5	26.7 ± 1	11.3 ± 1
5% Ethanol-insoluble residue of oven-dried alfalfa meal	—	—	—	—	8.0(8)	78.0 ± 5 *	25.4 ± 2	11.6 ± 1
0.1% rutin in diet	—	—	—	—	8.0(4)	81.9 ± 6	39.0 ± 2	17.5 ± 1
30 mg rutin per os daily	—	—	—	—	7.7(8)	89.3 ± 8	34.3 ± 2 *	15.5 ± 2
0.01% α-tocopheryl acetate	—	—	—	—	7.8(8)	63.1 ± 5	24.2 ± 3	9.3 ± 1

[1] Soybean protein basal diet with an oral supplement of 30 mg ascorbic acid/animal daily unless other ascorbic acid supplementation is specified. Values are mg ascorbic acid per 100 g fresh tissue as determined by 2,4-dinitrophenylhydrazine method.

[2] Daily gain for 4 weeks (number analyzed).

[3] SE of mean.

[4] Significantly different from basal, * $P < 0.05$; ** $P < 0.01$.

TABLE 5

Failure of level or frequency of ascorbic acid supplementation to affect response to alfalfa

Oral ascorbic acid supplement [1]	Daily gain, 4-wk Alfalfa supplement			
	None		5%	
	No.		No.	
		g		*g*
Males				
5 mg daily	12	7.5 ± 0.20 [2]	8	8.1 ± 0.32
10 mg daily	12	7.5 ± 0.25	12	8.4 ± 0.26 * [3]
20 mg daily	12	7.2 ± 0.27	8	8.3 ± 0.36 *
30 mg daily	24	7.4 ± 0.20	28	8.2 ± 0.16 **
Females				
30 mg daily	24	6.2 ± 0.18	24	7.2 ± 0.20 **
30 mg every 3rd day	15	6.1 ± 0.27	16	7.1 ± 0.25 **
30 mg weekly	15	6.0 ± 0.18	15	6.8 ± 0.20 **

[1] Soybean protein basal diet with ascorbic acid omitted.
[2] Standard error of the mean.
[3] Significantly different from the non-alfalfa supplemented group, * $P < 0.05$; ** $P < 0.01$.

centrations of adrenal ascorbic acid. The bioflavonoid, rutin, also produced an increase in the ascorbic acid content of adrenal and spleen, but additional α-tocopheryl acetate had no effect.

That the response to alfalfa does not depend upon the level or frequency of single ascorbic acid doses is shown in table 5. The growth of males fed the basal diet was not influenced by daily oral doses of ascorbic acid ranging from 5 to 30 mg. At each level of ascorbic acid, however, there was a significant response to the addition of 5% oven-dried alfalfa. When the ascorbic acid was given to females at intermittent periods, up to intervals of one week, they grew at a greater rate when the alfalfa supplement was added to the basal and there was no difference in the response obtained.

Growth rates obtained in the bioassay of the oven-dried alfalfa for vitamin C are shown in figure 1. Guinea pigs fed 5% alfalfa were growing at a slightly faster rate after 2-weeks than those fed the unsupplemented scorbutogenic diet, but thereafter the growth rate of both groups decreased sharply. Less than 50% of either group survived for 4-weeks and none was continued longer on the scorbutogenic regimen. The average time (17 days) at which gross symptoms of scurvy developed was the same for both groups. The effect of supplemental oven-dried alfalfa on the development of scurvy when a suboptimal amount (0.5 mg per os daily) of ascorbic acid was given is also shown in figure 1. This dose is about one-half the requirement for the size of animals used (15). Animals fed the alfalfa-supplemented diet consistently exhibited a greater growth rate than those fed the unsupplemented basal, and the average length of time for the appearance of gross scurvy symptoms was prolonged by about 10 days.

DISCUSSION

From a survey of adequate diets, Reid (16) suggested 20 mg of ascorbic acid per 100 g of diet as the minimal ascorbic acid requirement of the guinea pig. Collins and Elvehjem (15) reported that 0.5 mg of ascorbic acid per 100 g body weight is adequate for growth and verified the value by tissue analysis. The basal diets used in the present study contained initially 200 mg of ascorbic acid per 100 g, and when ascorbic acid was given orally as a single dose, each animal received 30 mg per day. Higher oral doses, up to 60 mg per day, did not improve the growth rate. It is significant that ad libitum consumption of an ascorbic acid solution stimulated the growth to a rate above that observed when single daily doses were administered. It is possible that continuously available ascorbic acid, either in the form of cabbage or ascorbic acid solution, maintains a critical level of ascorbic acid in some biochemical compartment. Penney and Zilva

(17) showed that 25 mg of ascorbic acid given orally as a single dose, resulted in tissue ascorbic acid levels that were less than one-half of those observed when the 25 mg were given in 50 doses at 10-minute intervals. There was no evidence that alfalfa spares the minimal ascorbic acid requirement, but it may contain a vitamin C-economizing factor (6, 7) in the sense that it elevates adrenal ascorbic acid slightly.

The insoluble alfalfa component stimulates growth when ascorbic acid is supplied as a single daily dose and gives about the same response as ad libitum ascorbic acid solution. All attempts to solubilize the factor have been unsuccessful. Exhaustive extraction with water, alcohol and petroleum ether did not remove the active component. The organic solvents removed reducing substances equivalent to 50 mg of α-tocopherol, but the extracts were inactive. It was determined, by both chemical and biological assay, that the active component in the oven-dried alfalfa meal is not ascorbic acid. One explanation for the relationship between the alfalfa factor and ascorbic acid is that the alfalfa components spare ascorbic acid. The converse hypothesis is as reasonable, namely, that ascorbic acid spares the requirement for a nutrient supplied by the alfalfa. Analogies to the latter postulate are known, for example, the vitamin-sparing effects of ascorbic acid in the rat (18, 19).[5] It is known that bulk stimulates the growth rate of guinea pigs (20) but the factor under study cannot be identified with cellulose. The basal diet contains 15% cellulose, and 5% more had no effect on the growth rate. Since it has not been possible to identify the alfalfa residue component with any known nutrient, it is postulated that this is an unrecognized growth stimulant. It appears to be metabolically related to ascorbic acid, but this may be by way of the intestinal flora.

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Mechanism of Appetite Control in Rats Consuming Imbalanced Amino Acid Mixtures

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ABSTRACT The hypothesis was tested that reduced food intake following ingestion of an imbalanced amino acid mixture is a consequence of abnormally low tissue concentrations of the free limiting amino acid. Rats were fed diets containing only L-amino acid mixtures as their protein source. Groups of animals received ad libitum a basal diet, a histidine-imbalanced diet, or a corrected diet. Controls included the histidine-imbalanced diet force-fed to match the ad libitum consumption of the corrected diet and the corrected diet pair-fed to match the ad libitum consumption of the imbalanced diet. In experiments of this type free concentrations of all amino acids were determined at short time intervals in liver, muscle, and plasma, and food consumption was measured. Marked depression of the concentration of the limiting amino acid occurred in 3 to 5 hours, the reduction in diet consumption appearing some 20 hours later. In experiments where animals that were adjusted to an imbalanced mixture were supplemented with the limiting amino acid, there was again a lag of 18 to 20 hours between correction of the tissue levels of the free limiting amino acid and an increase in diet consumption. These data suggest that it is not tissue levels of free limiting amino acid per se that suppress or stimulate intake of the imbalanced diet, but rather some metabolic consequence of these tissue concentrations. Growth experiments established that almost all the growth depression resulting from the imbalanced group is accounted for by the reduced food intake.

A series of investigations by Harper and his colleagues (1–13) led to formalization of their thinking about the sequence of events leading to the reduction in food intake and consequent growth failure that are characteristic of the amino acid imbalance phenomenon (14–16).

The basic elements of the rationale are that on ingestion of an imbalanced amino acid mixture the tissues are presented with an array of amino acids in which one or more amino acids are in short supply relative to the others. Presented with this pattern of protein precursors, the tissues synthesize protein at normal or nearly normal rates, thus driving the equilibrium concentration of the limiting amino acid to very low values. These depressed concentrations of the limiting amino acid in the tissues are soon reflected in the amount of circulating limiting amino acid. Eventually a lower threshold concentration is reached at which an unspecified appetite control mechanism is triggered to suppress further eating of the diet.

Portions of this hypothesis have since been tested experimentally and found to be valid. In particular, isotopic evidence using ^{15}N and ^{14}C has been presented demonstrating that protein synthesis from an imbalanced diet does proceed at a normal or supernormal rate (16, 17). As a consequence the retention efficiency of the limiting amino acid is very high (17), and low concentrations of the limiting amino acid in the blood and tissues appear quickly after ingestion of an imbalanced mixture.

The present report presents data clarifying the histidine imbalance system that this laboratory has been using (17) as well as new data which indicate that the appetite control involved in amino acid imbalance probably responds to some metabolic consequence of the low concentrations of the limiting amino acid rather than to these low values per se.

METHODS

To have maximal experimental flexibility, diets were developed in which all protein was supplied in the form of L-amino acids. The basis for the amino acid mix-

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tures used here was the set of diets described by Sauberlich (18).

The basal diet consisted of the following per kilogram of diet: (in grams) 100, corn oil; 0.5, cod liver oil; 0.23, α-tocopheryl acetate; 0.006, menadione; 2.0, choline chloride; 1.0, *myo*-inositol; 50, salt mixture no. 5 of Salmon (19); 50.145, sucrose-vitamin mixture; 103.25, amino acid mixture H of Sauberlich (18); and 692.869, sucrose. This quantity of sucrose-vitamin mixture provided the following: (in milligrams) 2.0, folic acid; 0.05, vitamin B_{12}; 29.0, niacin; 35.0, Ca-pantothenate; 7.0, thiamine·HCl; 7.0, riboflavin; 14.0, pyridoxine·HCl; 0.6, biotin; and 50.0, ascorbic acid. The amino acid mixture contained the following as L-isomers per kilogram of diet: (in grams) 3.00, alanine; 4.00, arginine·HCl; 3.00, aspartic acid; 3.0, asparagine monohydrate; 1.50, cystine; 20.00, glutamic acid; 2.00, glycine; 2.50, histidine·HCl; 10.00, leucine; 7.50, isoleucine; 9.25, lysine·HCl; 4.00, methionine; 3.25, phenylalanine; 2.50, proline; 2.50, serine; 3.75, threonine; 2.50, tryptophan; 4.0, tyrosine; 7.00, valine; and 8.00, $NaHCO_3$. This mixture contains only half as much histidine as Sauberlich's mixture H (18) and is satisfactory for creating a histidine imbalance. In certain experiments the preceding amounts of arginine, cystine, leucine, isoleucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine and 3.20 g of $NaHCO_3$ were used as mixture A, and the corresponding amounts of the remaining amino acids (nonessential amino acids plus lysine) were used as mixture B.

All components and diets were stored at 4°, and fresh vitamin mixtures were prepared every 3 months. Amino acid mixtures to yield imbalanced and corrected diets were added to the basal diet at the expense of sucrose. The purity of all amino acids was determined to be in excess of 99% by ion exchange chromatographic analysis (20). No trace of the *allo* isomers of threonine and isoleucine could be found.

Male weanling rats of the Sprague-Dawley strain were housed individually in stainless steel cages with unlimited access to water and food except when either paired feeding or forced feeding was used. All animals were fed the basal diet for 3 to 5 days before the experiments were started. Animals showing satisfactory gains and food consumption were then grouped for uniformity in weight into groups of at least five and fasted for 12 to 15 hours before the experiments were started. In experiments in which animals were killed at short intervals, both the experimental animals and their controls were kept in continuously lighted rooms.

Food intake was measured over intervals of several hours for the first 2 days and over daily intervals thereafter. Any exceptions to this protocol are noted later at the appropriate point. Pair-fed and force-fed control groups were used frequently. For force-feeding it proved impossible to prepare satisfactorily stable emulsions from the diet in its usual form. Therefore, for force-feeding a suspension of the water-soluble components of the diet was blended in a minimal amount of water until the suspension was uniform. One milliliter of this suspension contained 0.8 g of diet. The fat-soluble components were then fed separately. A size 8 catheter with a calibrated syringe was used in all force-feeding. The corn oil-fat-soluble vitamin mixture was fed once daily, and the water-soluble components were fed in 2- and 3-ml portions 4 to 6 times a day, spaced equally over an 18-hour period.

To obtain specimens for analyses of free amino acids, animals were anesthetized with ether and exsanguinated in groups of three. Systemic blood, gastrocnemius muscle and liver specimens were then pooled. After centrifugation, plasma was stored at 0°. Liver and muscle samples were frozen immediately in an ethanol-dry ice mixture and then stored at 0°. They were blotted and weighed just before further preparation.

Tissue samples were homogenized in 10 volumes of 1% picric acid (21) with the use of either an all-glass homogenizer or a high-speed blender. Protein precipitates were removed by centrifugation and the supernatants were freed of excess picric acid by passage through a weak anion exchange resin in the chloride cycle. Col-

umns that were 2 cm × 10 cm could be reused 2 or 3 times provided they were regenerated after each use with 1 N HCl and then washed with water until chloride-free. Samples were eluted with water followed by 0.02 N HCl, and the combined effluents were concentrated in vacuo, adjusted to pH 7.0 to 7.2, and treated with an excess of sodium sulfite at room temperature for 4 hours to convert glutathione to glutathione-S-sulfonate. The extracts were then diluted to the desired volume with buffer at pH 2.2 and analyzed in duplicate.

Plasma samples were similarly processed except for deletion of the sulfite step.

Amino acid analyses were carried out as described by Spackman et al. (20) and Moore et al. (22). Internal standards were used with all samples, norleucine for the neutral and acidic amino acid column and guanidino propionic acid for the basic amino acid column.

RESULTS AND DISCUSSION

General characteristics of this imbalance system are described by the data in tables 1, 2, and 3. The data in table 1 are from an experiment in which a histidine imbalance was created. To produce an imbalanced diet the basal diet was modified by adding an additional 10% of the amino acid mixture minus histidine. A corresponding corrected diet was prepared by restoring the histidine to its original level. All animals were fed the corrected diet for 2 days. They were then divided into 2 paired groups of seven according to body weight, and the experimental diets were offered ad libitum. Growth and food consumption were observed. These data show that the imbalanced mixture resulted in reduced food intake within 24 hours after its introduction, the food consumption of the imbalanced diet during the first day after that period being only 59% of that for the corrected diet. After introduction of the imbalanced mixture food intake was normal for the first 8 hours; but between the eighth and the twenty-fourth hour it fell to 2.5 g compared with 4.8 g for the controls receiving the corrected diet, a difference that was highly significant statistically. The reduction in intake, then, failed to occur until at least 8 hours after introduction of the imbalanced mixture. Subsequently growth ceased abruptly and remained negligible over the following week. The system, then, shows quite rapidly the animal response that is characteristic of amino acid imbalances. The effect is further clarified by noting that the protein efficiency ratio (PER) of the imbalanced mixture was only 0.45, whereas that of the corrected diet was 3.4.

TABLE 1

Growth and food consumption of rats fed imbalanced and corrected amino acid mixtures

Day [1]	Food consumption		Body wt	
	Imbalanced	Corrected	Imbalanced	Corrected
	g	*g*	*g*	*g*
−2	2.8	2.4	43	37
−1	4.0	3.9	—	—
0	—	—	47	39
1	3.6	6.1	46	43
2	1.3	6.4	45	47
3	2.0	6.2	45	51
4	2.2	6.7	46	55
5	3.1	7.6	44	59
6	3.5	7.9	47	66
7	3.7	8.6	48	72

[1] On days −1 and −2 animals were fed the corrected diets. Days 1, 2, 3, etc., refer to time since introduction of experimental diets. All values are the average of 7 animals/group.

Table 2 describes a series of diet formulations designed to simplify the amino acid mixture used in creating an imbalance. As mentioned earlier, mixture A consisted of essential amino acids only, and mixture B consisted of lysine plus the nonessential amino acids in the proportions used in preparing the basal diet.

Animal performance with these diets is shown in the 2 experiments in table 3. Interval feeding, as in the first trial, resulted in considerably higher food consumption and PER for imbalanced diets than ad libitum feeding, confirming that imbalanced amino acid mixtures are within the limits of metabolic accommodation provided they are eaten in sufficient quantities. Of particular importance here is the comparison of the performance of group 1 with groups 3 and 5 for these define clearly the basic properties of the

TABLE 2

Composition of diets used in simplifying amino acid mixtures used to create imbalance for histidine

Diet No.	Diet Designation	Addenda to basal [1] diet [2]	Histidine content of diet
			%
1	10% basal [3]	none	0.25
2	10% basal + histidine	0.25% histidine	0.50
3	20% imbalanced	10% mixture H less histidine	0.25
4	20% corrected	10% mixture H less histidine + 0.75% histidine	1.00
5	15% imbalanced, A	5% mixture A	0.25
6	15% corrected, A	5% mixture A + 0.75% histidine	1.00
7	15% imbalanced, B	5% mixture B	0.25
8	15% corrected, B	5% mixture B + 0.75% histidine	1.00
9	15% basal	5% mixture H, minimal histidine	0.375
10	15% basal + histidine	5% mixture H, minimal histidine + 0.35% histidine	0.725

[1] Composition of basal diet and mixtures H, A, and B are given in text.
[2] Addenda were at expense of sucrose.
[3] 10%, 15% and 20% refer to the protein concentration in the diet.

TABLE 3

Growth and food consumption of rats consuming a series of diets imbalanced or corrected with respect to histidine

Diet		Trial 1 [1]			Trial 2 [2]		
No.	Designation	Food eaten	Wt gain	PER [3]	Food eaten	Wt gain	PER
		g/7 days	*g*		*g/5 days*	*g*	
1	10% basal	39.3	13.7	3.5	53.1	18.5	3.5
2	10% basal + histidine	46.9	14.7	3.1	53.6	17.8	3.3
3	20% imbalanced	35.2	13.7	2.0	31.5	4.8	0.74
4	20% corrected	40.1	29.5	3.3	56.4	36.4	3.2
5	15% imbalanced, A	35.1	12.7	2.4	29.1	2.4	0.54
6	15% corrected, A	52.0	33.3	4.3	56.6	27.2	3.1
7	15% imbalanced, B	30.1	7.0	1.7	—	—	—
8	15% corrected, B	35.5	10.3	1.9	—	—	—
9	15% basal	32.7	11.7	2.7	29.5	3.6	0.82
10	15% basal + histidine	52.1	29.7	3.8	54.8	29.0	3.54

[1] Mean values are given for groups of 3 rats trained for 3 days to eat 2 meals daily during feeding periods of 1 hour each. Trial 1 was thus with interval-fed animals, and trial 2 was with ad libitum-fed animals.
[2] Mean values for groups of 5 rats except group 1 which had 10 rats. All animals were fed ad libitum.
[3] PER represents grams of gain per gram of protein eaten.

imbalance system being studied. Mixture A, containing all of the essential amino acids except lysine, was used to create an imbalance in most of the subsequent research because it was readily corrected by histidine, whereas the adverse effects caused by supplementing the basal diet with mixture B were not. Two critical types of control have been used in this experiment, the basal diet group 1 and the corrected diet group 6.

Further attempts to simplify the mixture of amino acids used to induce imbalance in the basal diet resulted in only weak imbalance responses. Supplementation with threonine or with methionine plus phenylalanine was evaluated and yielded histidine-correctable growth depressions, but these growth depressions were mild and not judged suitable for study of the short-term sequence of events following introduction of imbalanced mixtures.

The minimal amount of histidine needed to correct the histidine imbalance resulting when 5% of mixture A, the essential amino acids, was added to the basal diet, led to the results shown in table 4. In this experiment histidine was added to the imbalanced diet at concentrations of

TABLE 4
Alleviation of amino acid imbalance by histidine [1]

Histidine		Wt gain	PER [2]	HER [3]
Added to imbalanced diet	Total in diet			
%	%	g/6 days		
None	0.25	9.8	1.73	106
0.125	0.375	37.4	4.07	163
0.25	0.50	39.8	4.23	128
0.50	0.75	41.8	4.09	87
0.625	0.875	38.2	4.03	69
0.75	1.00	39.6	4.06	61

[1] Five rats/group.
[2] PER represents grams of gain per gram of protein eaten.
[3] HER represents grams of gain per gram of histidine eaten.

zero, 0.125, 0.25, 0.50, 0.625, and 0.75%. Groups of five weight-paired weanling rats were offered the diets ad libitum for 6 days. Weight gains and food consumption were measured yielding the data in table 4.

The optimal histidine content varied depending upon the parameter. Total gain reached a maximal value at 0.50 to 0.75% total histidine and remained nearly constant at values up to 1.00% total histidine. The PER reached a maximum at 0.50% histidine and dropped to slightly lower values at higher levels of histidine. Optimal efficiency of histidine utilization, shown in table 4 as HER (grams of gain per gram of histidine eaten), occurred at somewhat lower levels (0.375% histidine) and fell markedly as the histidine level rose. Considering all 3 parameters, it appears that the least amount of histidine giving full correction of the imbalance is in the range of 0.375 to 0.50%. This level of total histidine is achieved by adding only 0.125 to 0.25% histidine to the imbalanced diet.

It is implicit in the Harper hypothesis that growth failure with imbalanced amino acid mixtures is a secondary effect of the reduced food consumption — that animals consuming an imbalanced mixture should grow relatively well if their consumption is artificially maintained. Adherence of the present experimental system to these conditions is demonstrated by the data in table 5. In this experiment groups of 6 animals were fed ad libitum the 10% protein basal diet, the 15% protein imbalanced diet, and the 15% protein corrected diet. A fourth group was given the corrected diet pair-fed to the intake of the group consuming the imbalanced diet ad libitum. A fifth group was force-fed the imbalanced diet to match the consumption of the corrected diet eaten ad libitum.

Restriction of intake of the corrected diet resulted in depressions of gain and PER to values approaching those of animals consuming the imbalanced diet ad libitum. Force-feeding of the imbalanced mixture caused marked improvement in both gain and PER, surpassing values obtained with the basal diet and approaching the values resulting from feeding the corrected diet ad libitum.

To assess the time-sequence of the food intake reduction and changes in tissue concentrations of free amino acids, groups of 3 animals were killed at each time-interval and duplicate analyses of pooled samples were made except on plasma on which only single analyses were made. Feeding schedules were the same as those for groups A, B, C, and D

TABLE 5
Food consumption and growth response of animals consuming balanced and imbalanced amino acid mixtures [1]

Group	Diet	Feeding schedule	Food intake	Wt gain	PER [2]
			g/7 days		
A	15% corrected	ad libitum	76.4	37.7	3.3
B	15% corrected	pair-fed to group D	51.5	18.5	2.4
C	10% basal	ad libitum	78.1	26.7	3.4
D	15% imbalanced	ad libitum	52.1	15.3	2.0
E	15% imbalanced	force-fed to group A	72.7	30.3	2.8

[1] Six animals/group.
[2] Grams of gain per gram of protein eaten.

of table 5. Complete data for all free amino acids in liver, muscle, and plasma have been obtained,[2] but here only representative curves have been selected for inclusion.

Fluctuations in the concentrations of free histidine are of particular interest and appear in figure 1. The 3 tissues showed comparable trends in all 4 groups. The absolute fluctuations were largest in liver and smallest in plasma, although expressed as percentages of the fasting values the fluctuations are similar in magnitude. With ad libitum consumption of the imbalanced mixture marked depression of free histidine concentration below fasting values was noted after 4 hours

[2] Ellison, E. J. 1967 Studies of an acute amino acid imbalance in the rat, Ph.D. Dissertation, Virginia Polytechnic Institute, Blacksburg, Virginia.

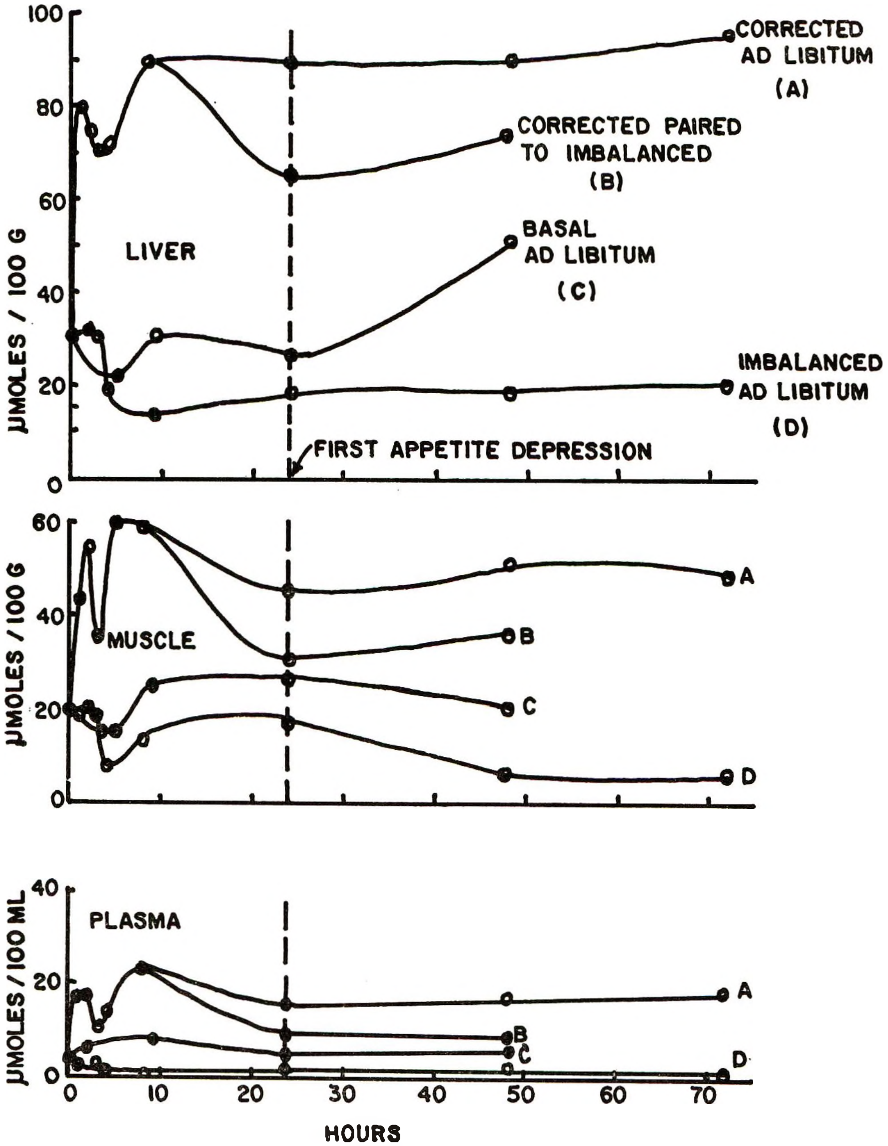

Fig. 1 Free histidine levels in tissues of rats fed basal, imbalanced and corrected amino acid mixtures.

and persisted throughout the 72-hour period. These values were distinctly lower than the concentrations with the basal diet or either of the corrected diets. It is assumed that the fluctuations in histidine concentrations between the second and the eighth hours resulted from heavy food consumption following the 12-hour fasting period. In contrast with the early depression in free levels of histidine which was clear-cut at 4 hours, appreciable reduction in food intake did not appear until 24 hours after introduction of the diets. The significance of this lag will be considered later.

The changes in concentration of free plasma leucine (see fig. 2), isoleucine, and valine were similar. During the initial 8 hours (the critical period from an interpretive standpoint because changes then have not been confounded by the effects of food intake) the aliphatic essential amino acids tended to be lower in concentration in animals consuming the imbalanced diet compared with those consuming the corrected diet. None of these changes, however, compares in magnitude with those of histidine.

In general, with all amino acids the animals receiving the corrected diet with its intake paired to that of the imbalanced group, showed only partial modification of the fluctuations in amino acid concentration, which indicates that the changes occurring during the first 8 hours reflect

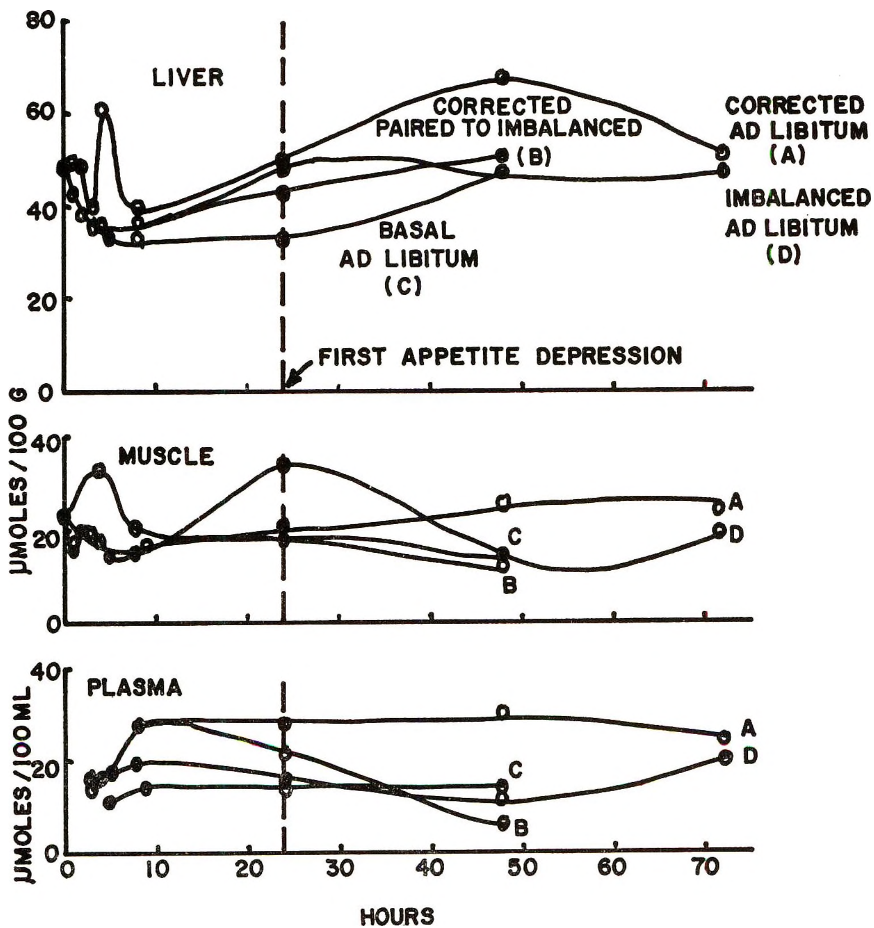

Fig. 2 Free leucine levels in tissues of rats fed basal, imbalanced and corrected amino acid mixtures.

the imbalance per se rather than the reduction in food consumption.

In all treatments except for the basal group the concentration of lysine in all 3 specimens declined steadily over the first 8 hours, the imbalanced groups showing the slowest decline. Plasma glycine concentrations tended to be highest in the imbalanced group. Plasma glutamic acid was lowered in the ad libitum imbalanced group in comparison with the ad libitum corrected group within 3 hours. The data for liver and muscle showed no such distinct differences.

For the most part the inter-group differences for the other amino acids were noted after 24 hours, and cannot therefore be attributed to the imbalance of amino acids alone.

The rapid and severe depressions in liver, plasma, and muscle concentrations of histidine demonstrated here confirm in a new experimental system the same phenomenon observed when other imbalanced amino acid sources were used. The data also support Harper's hypothesis that depression in the tissue concentrations of the limiting amino acid are a very early consequence of the ingestion of an imbalanced amino acid mixture. Two further points are clarified by the data. One is that tissue levels of other amino acids are disturbed as a consequence of the imbalance. The second is that there is a lag of approximately 20 hours between full expression of the lowered concentrations of the limiting amino acid and the suppression of eating.

That a time-lag of similar duration occurs between restoration of normal free histidine levels and the resumption of eating, is shown by the following experiment. Two groups of 14 animals were offered the imbalanced or the corrected diet ad libitum for 75 hours at which time the depression in intake was complete. The 14 animals receiving the imbalanced diet were divided into 2 groups of seven. One of these groups was force-fed 100 mg of histidine in solution and the other the same volume of water. This amount of histidine was approximately that being consumed daily by the control group receiving the corrected diet ad libitum. It was given as a 10% aqueous solution of histidine hydrochloride adjusted to pH 7.0. Food consumption and plasma, liver and muscle concentrations of free amino acids were then followed at short intervals for an additional 30 hours. The results on food intake appear in table 6, and show that the supplemented animals had increased their intake of imbalanced diet approximately 18 hours after the supplement was given.

Tissue histidine concentrations from this same study appear in figure 3. Following supplementation there was an immediate sharp rise in free histidine levels in all 3 tissues to values that were well above the normal values observed in the tissues of animals receiving the corrected diet. In general, histidine levels returned to subnormal values within 16 hours. Food consumption was not increased until 18 hours after the supplement when free histidine levels in liver and plasma had already returned to the very low levels characteristic of the imbalanced diet.

Concurrent changes in the levels of leucine, isoleucine and valine again followed similar courses, the valine data being shown in figure 4. The changes are not comparable to those in histidine, but in general there is a transitory drop in concentration immediately following the histidine supplement, followed by a return toward normal values similar to those observed in animals fed the corrected diet.

TABLE 6

Ad libitum food intake of rats consuming an imbalanced mixture of amino acids before and after histidine supplementation

Time from start of experiment	Ad libitum food intake	
	Supplemented group	Non-supplemented group
hours	*g/hr*	*g/hr*
0–24	0.333	0.306
24–48	0.276	0.283
48–75 [1]	0.272	0.296
75–80	0.460	0.490
80–85	0.240	0.320
85–93	0.288	0.338
93–99	0.332 *	0.200
99–105	0.543 *	0.351

[1] Supplement was administered at the seventy-fifth hour.

* Difference highly significantly different between groups at $P < 0.01$ in t test; values are averages of 2 rats/group.

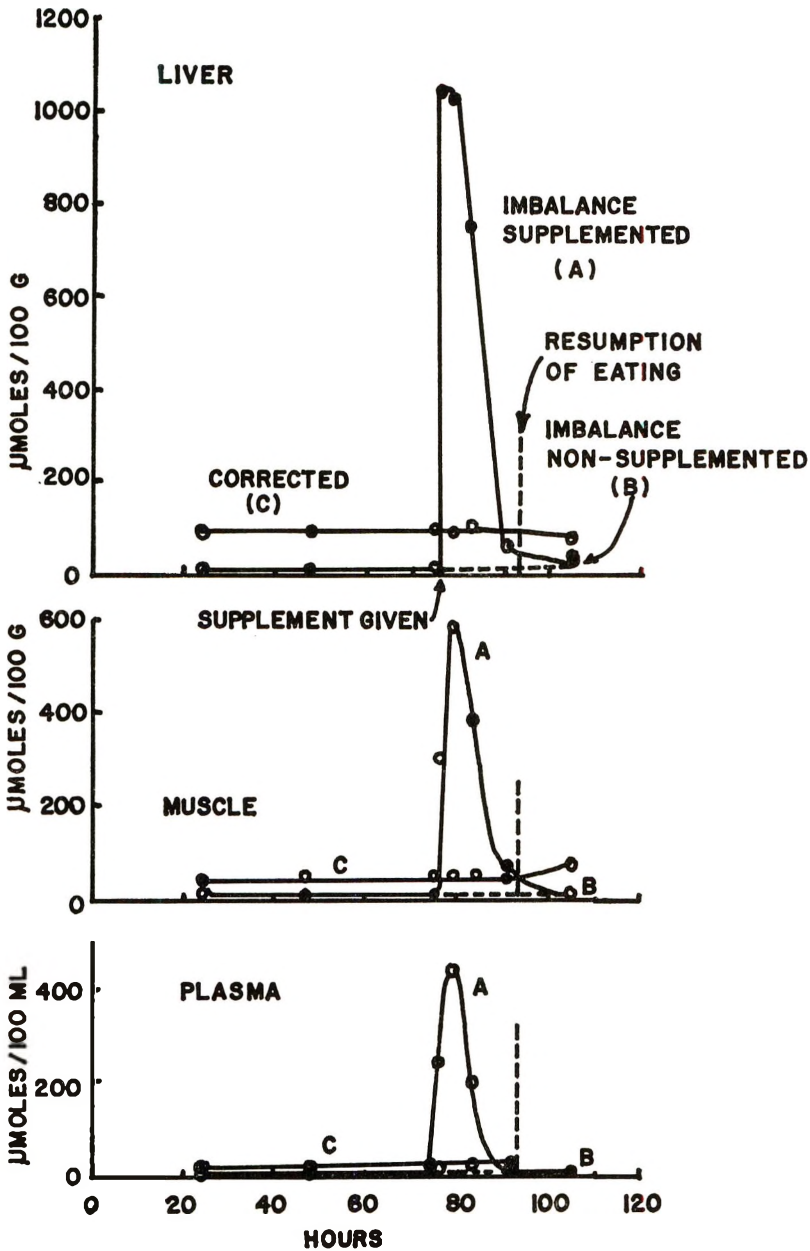

Fig. 3 Tissue concentrations of histidine as affected by histidine supplementation of rats fed an imbalanced amino acid mixture.

The exceptions to this transitory drop were observed with glutamic acid, glycine, proline, and alanine and were particularly marked in plasma in contrast with liver, for which depressions usually occurred. The alanine response in plasma is shown in figure 5 and appears to reflect clearly the catabolism of the excess histidine supplement.[3]

Considering these data it appears likely that Harper's hypothesis is basically valid

[3] Detailed data on all the other amino acids are to be found in the doctoral dissertation on which this report is based (see footnote 2).

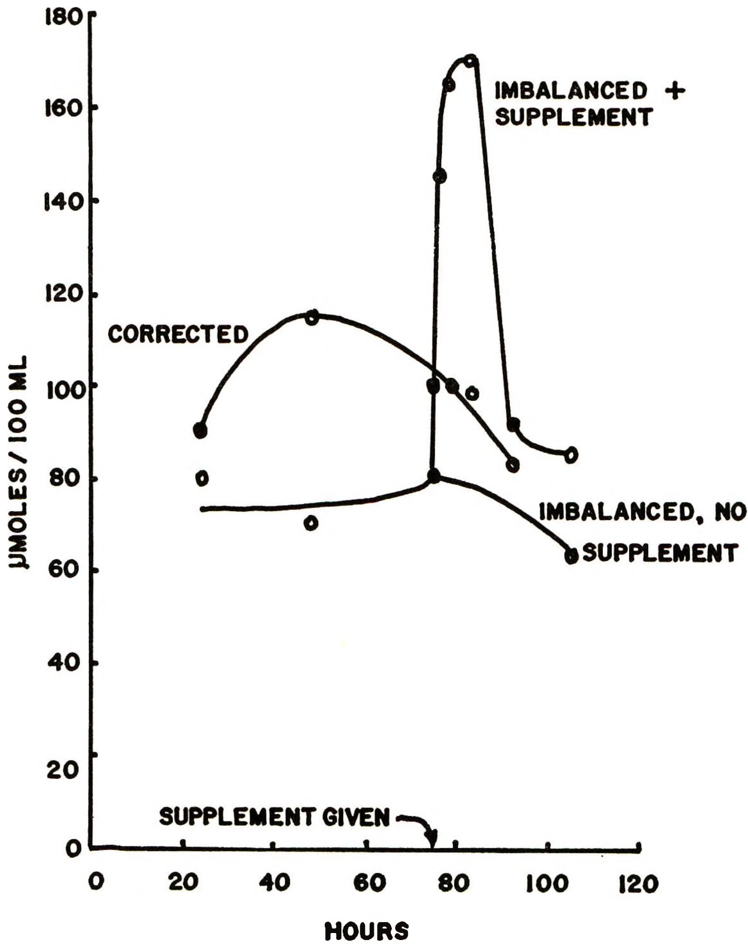

Fig. 4 Effect of histidine supplementation on plasma alanine concentrations after supplementing rats fed a histidine-imbalanced amino acid mixture.

— that severe depression of the concentration of the limiting amino acid in the tissues following introduction of an imbalanced diet results in depressed diet consumption. The much smaller changes in concentration of other free amino acids appear to be secondary or side events. It appears unlikely, however, that it is the concentration of the limiting amino acid per se that actually suppresses, or, after supplementation, restimulates food intake. This conclusion is based on the 8- to 24-hour lag between the drop in histidine levels and the curtailment of eating and by the similar lag between the high histidine levels following supplementation and the increased intake of imbalanced diet.

This lag suggests that perhaps the synthesis of some proteinaceous appetite regulator is retarded by the histidine deficit, a lapse of 8 to 24 hours being required to exhaust the already synthesized reserves. Similarly in the animal with an established suppression of appetite caused by a past history of eating an imbalanced mixture, some 18 hours are required before the resynthesis made possible by supplementation can cause accumulation of enough appetite stimulator to effect the increase in intake.

There is obvious contradiction between this suggestion and that of Davis et al. (23) that appetite suppression can be the result of the presence of a humoral satiety

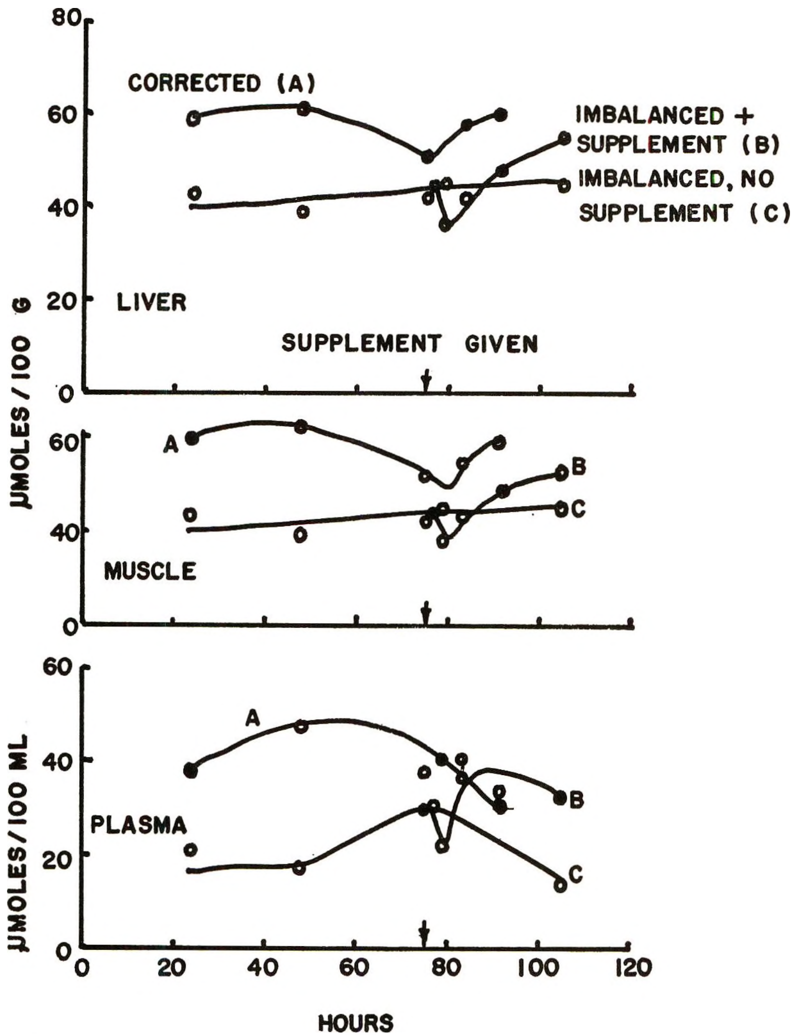

Fig. 5 Tissue concentrations of free valine as affected by histidine supplementation of rats fed an imbalanced amino acid mixture.

factor following eating. In defining the area of disagreement 2 points warrant particular attention. One is that there is positive evidence supporting the present suggestion, whereas Davis et al. (23) cite only negative observations for the absence of such a factor in the fasted animal. The second is that there are marked differences in the two experimental systems, this study dealing with the relatively specific effects of amino acid imbalance and the other with the quite general effects of consumption of a sweetened condensed milk. The entire discrepancy may arise from this latter difference in the type of appetite control operating in the two experimental systems.

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