Ultrastructure of Aortic Tissue in Copper-deficient and Control Chick Embryos [1,2]

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ABSTRACT Studies were conducted to investigate ultrastructure of aortic tissue of copper-deficient and control chick embryos. The copper-deficient and control embryos were obtained by feeding hens purified diets containing 2 and 46 ppm copper, respectively. The aortas of these 18-day embryos were studied by light and electron microscopy. The tunicae media of control embryos consisted of alternating layers of cells which were separated by narrow extracellular spaces containing elongated strands of elastic fibers. By electron microscopy, it was observed that these elastic fibers had smooth contours and consisted of parallel fibrils embedded in a matrix of low electron density. The tunicae media of copper-deficient embryos consisted of an unorganized population of cells which were separated by wide extracellular spaces containing elastic fibers in the form of globules or short strands. By electron microscopy, it was observed that these altered elastic fibers had knobby contours and consisted of few scattered fibrils embedded in an electron opaque matrix. As compared with controls, it appeared that cells in the tunicae media of copper-deficient embryos were immature, and the elastin synthesized was abnormal.

Mortality from angiorrhexis occurs in chicks (1) and young swine (2) fed a copper-deficient diet. Such vascular disease is characterized by connective tissue defects, especially fragmentation and disruption of elastic fibers in all tunicae of the involved artery. These elastic fiber changes in the aortas of chicks fed a copper-deficient diet, as observed by electron microscopy, have been described (3).

Recently it was suggested that the role of copper in the metabolism of aortic elastin was anabolic in nature (4). This report of the chemical analysis of aortas of chicks fed 0.8 and 25 ppm dietary copper and of related isotope experiments offered evidence that copper was required for the synthesis of normal elastin. It has also been observed that feeding of a low copper, non-fat milk-solids diet to hens causes production of copper-deficient embryos (5) which die in the early blood stages without evidence of gross abnormalities. Because of these reports, the present investigation was initiated in which copper-deficient embryos were produced and their aortas studied by light and electron microscopy.

EXPERIMENTAL

Thirty, 14-month-old, commercial egg-production type of hens (Hyline 934H) were assigned at random to 6 groups of 5 birds each, and maintained in individual cages. They had been fed a 17% protein practical-type diet composed of natural ingredients (6). Two groups were fed a purified diet (table 1) containing 2 ppm copper by analysis (7). Two other groups were fed the same purified diet supplemented with 46 ppm copper as anhydrous $CuSO_4$. The third 2 groups were continued with the practical-type diet and served as positive controls.

All hens were inseminated artificially with pooled semen at the start of the feeding trial and thereafter at 10-day intervals. Collection of eggs began 2 days after the initial insemination. The eggs were collected daily, stored at 13°, and set according to the date of lay at weekly intervals.

Eighteen-day, copper-deficient embryos from hens fed the purified diet containing 2 ppm copper were used for microscopic study. They were the last embryos that had lived to 18 days. Subsequent embryos died within the first 6 days of incubation. Control 18-day embryos were from hens fed the purified diet containing 46 ppm

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TABLE 1

Composition of purified diet [1]

	%
Non-fat milk solids	50.0
Sucrose	35.7
Corn oil	5.0
Glycine	0.5
DL-Methionine	0.3
Choline chloride	0.2
Micro-ingredients [2]	1.0
Salt mix [3]	7.3

[1] By analysis, diet contained 2 ppm copper.
[2] Vitamins added per 100 g of diet: vitamin A, 2000 USP units; vitamin D_3, 433 ICU; and (in milligrams) menadione, 2.5; α-tocopheryl acetate, 2.5; ethoxyquin (Santoquin, Monsanto Co., St. Louis), 0.0125; thiamine·HCl 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; niacin, 5.0; inositol, 50; biotin, 0.04; folic acid, 0.2; and vitamin B_{12}, 0.003.
[3] Salt mix: (in grams) $CaCO_3$, 500; $CaHPO_4$, 200; NaCl, 20; $ZnCO_3$, 4.5; $MnSO_4 \cdot H_2O$, 3.1; $FeC_6H_5O_7 \cdot 5H_2O$, 6.0; and KIO_3, 0.04.

copper. These were compared with embryos produced by hens that received the practical-type diet, and no differences were noted between the 2 groups.

A portion of the thoracic and abdominal aortas of 5 copper-deficient and 5 control 18-day embryos were examined microscopically. For histological studies, aortas were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 5 μ, and stained with hematoxylin-eosin stain and with Orcein-Van Gieson stain for elastic fibers. For electron microscopy, a small portion of the aortas was fixed in 2% glutaraldehyde in Sorenson's buffer, pH 7.2 (8), post-fixed in 1% OsO_4 (9), embedded in Araldite (10), sectioned, stained with lead citrate (11) and examined with a Philips EM200 electron microscope. One-micron sections of Araldite-embedded aortas were stained (12), and examined by light microscopy.

RESULTS

Feeding of the copper-deficient diet to laying hens resulted in mortality of all embryos within 10 days. However, some hens did not produce live embryos after receiving the deficient diet for only 4 days. This mortality was attributed to a copper deficiency, since no mortality was observed when the control diet was fed. Although hatchability was reduced to zero, egg production was not significantly affected. Egg production was slightly lowered in the groups receiving the diet containing 46 ppm copper, as compared with hens receiving the practical-type diet, but hatchability of fertile eggs was not affected.

Examination of most broken-out eggs indicated that they were fertile, but the embryo had died within the first 36 hours of incubation. The last embryo which survived beyond 36 hours usually died about the sixth day of incubation. The embryo produced prior to this one was alive at 18 days of incubation and was found to be copper-deficient by histopathologic studies.

When copper-deficient hens were returned to the practical-type diet, hatchability returned to normal in 18 days. The first surviving embryo at 18 days of incubation was copper-deficient according to histopathologic studies.

Light microscopy. The tunicae media of 18-day control chick embryos consisted of alternating layers of cells that were separated by narrow spaces (fig. 1). This extracellular space contained prominent, elongated strands of elastic fibers in the Orcein-Van Gieson stain, but not continuous elastic lamellae (fig. 2). These findings were also observed in 1-μ sections of Araldite-embedded control aortas. In 1-μ sections, most of the cells in the tunicae media of aortas of control embryos were spindle shaped and dark-staining (fig. 1A). Elastic fibers in the extracellular space were elongated strands which generally extended about the length of 2 confluent, longitudinally directed cells.

The tunicae media of the aortas of 18-day-old copper-deficient embryos differed from those of control embryos. The vascular wall of deficient embryos did not contain layers of cells. Instead, cells were observed as an unorganized population of cells, not closely associated with each other (fig. 3). As a result, the extracellular spaces were wider than those of controls. Moreover, elastic fibers in the extracellular spaces were fragmented, slightly swollen and consisted of short strands (fig. 4).

In 1-μ sections of copper-deficient embryos, most of the cells in the tunicae media were pale, round to oval in shape, and had pseudopod-like extensions (fig. 3A). The nuclei of these cells were large, had a vascular appearance, and their limiting membranes generally were smooth. Occasionally elongated, darker-staining cells having the characteristics of cells present

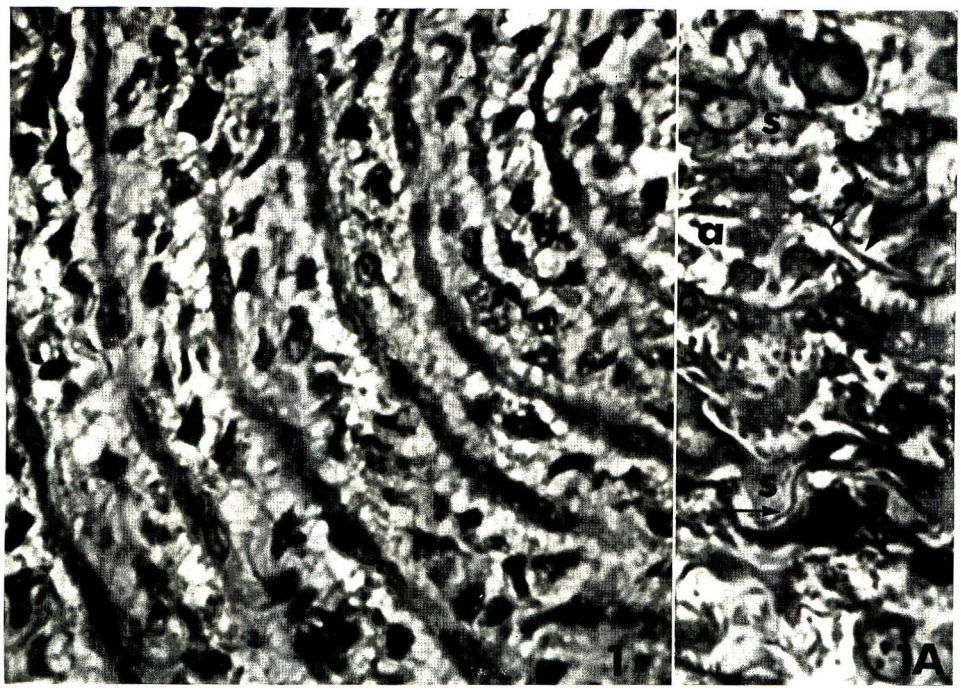

Fig. 1 Tunica media of the aorta of the control chick embryo contains alternating layers of cells. H&E. × 130. 1A: One-micron section, the cells in the media are spindle-shaped (S), and the extracellular spaces (a) are small. Continuous elastic fibers (arrows) are indicated by arrows. Toluidine blue. × 1200.

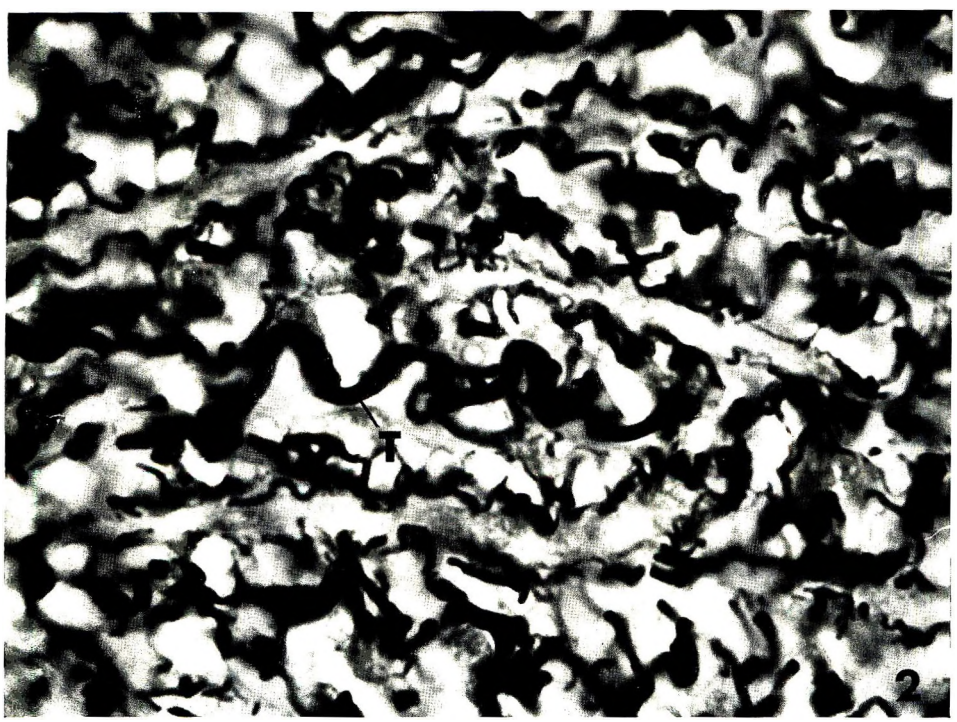

Fig. 2 Elastic fibers in the tunica media of the aorta of a control embryo consist of elongated strands (T). Orcein-Van Gieson stain. × 1200.

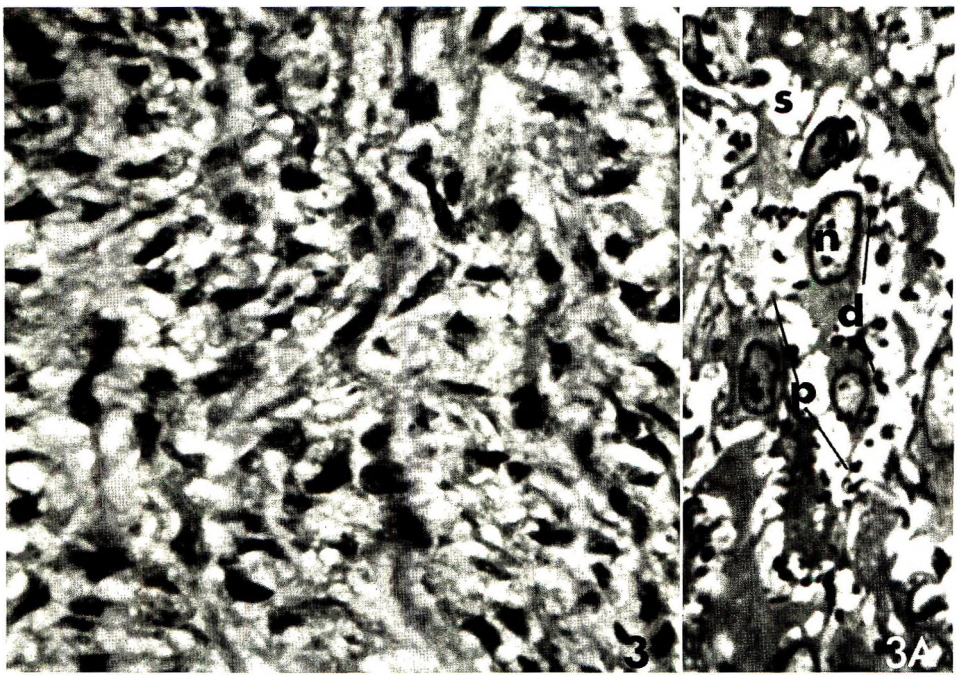

Fig. 3 Tunica media of the aorta of the copper-deficient embryo consists of an unorganized population of cells. H&E. × 130. 3A: One-micron section, the cells have processes (p), and the nucleus (n) is vesicular. The extracellular space (S) is dilated and contains elastic fibers in the form of droplets (d). Toluidine blue. × 1200.

Fig. 4 Elastic fibers in the tunica media of the copper-deficient aorta are broken strands (T) or droplets (D). Orcein-Van Gieson stain. × 1200.

in the aortas of control embryos, were interspersed between the lighter-staining, prevalent cell type. The extracellular spaces in the tunicae media were more extensive in deficient than control embryo aortas, and generally elastic fibers in this area were seen as small, dark droplets.

Electron microscopy. The tunicae media of the aortas of 18-day-old control chick embryos contained elongated cells. They were confined by a well-defined plasma membrane. Such cells contained irregular elongated nuclei having numerous indentations of the nuclear membrane, and they usually possessed prominent nucleoli (fig. 5). The cells also contained abundant, rough-surfaced endoplasmic reticulum (fig. 6). The cisternae of some endoplasmic reticulum appeared to be filled with amorphous material of low electron density, and many cisternae were parallel to the longitudinal axis of the elongated cell. Numerous mitochondria were also observed in these cells, but no filaments were observed. A distinct, but relatively small extracellular space separated most adjacent layers of cells in the tunica media. The space contained connective tissue consisting of elastic and collagenous fibers, delicate fibrillar elements, embedded in an electron translucent matrix. The elastic fibers were elongated strands in longitudinal section, had smooth contours and consisted of parallel fibrils embedded in a matrix of low electron density (fig. 6). Most of the elastic fibers were located close to the well-defined membranes of the spindle-shaped cells, or were in contact with the membranes. Collagenous fibers had periodicity. They were seen both in contact with the limiting membrane of the cells and free in the extracellular spaces.

The architecture of the tunicae media of the aortas of copper-deficient embryos did not resemble that of control embryos (fig. 7). Most of the cells in deficient em-

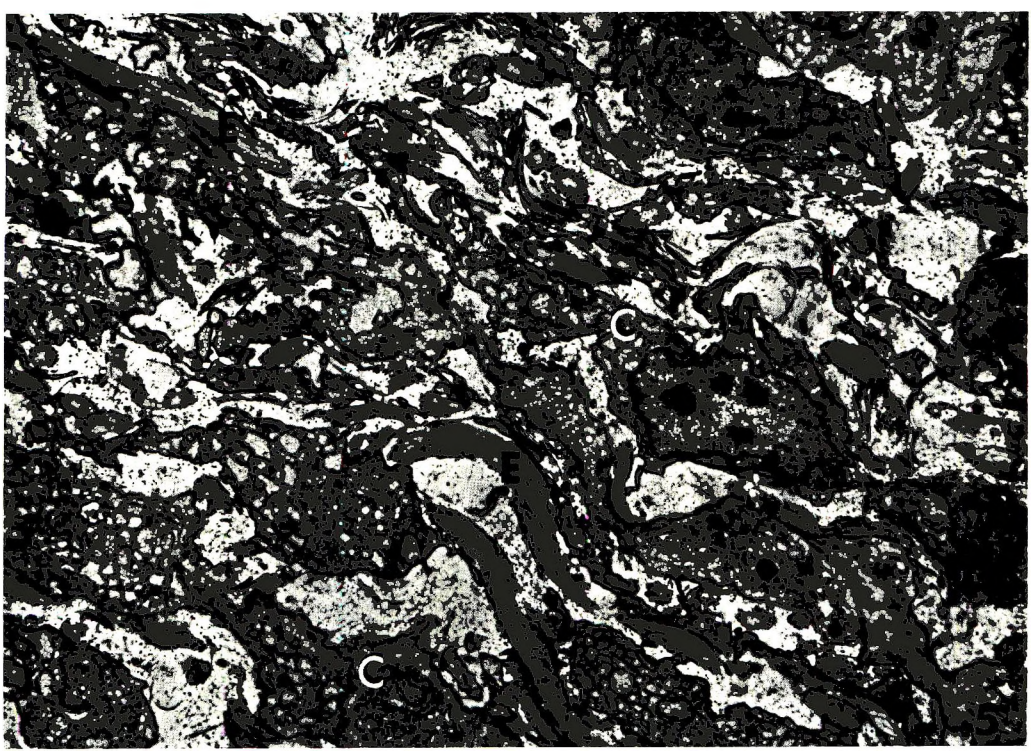

Fig. 5 Tunica media of the aorta of a control chick embryo. The spindle-shaped cells (C) are arranged in layers. Elongated strands of elastic fibers (E) are present in the extracellular space. × 8,000.

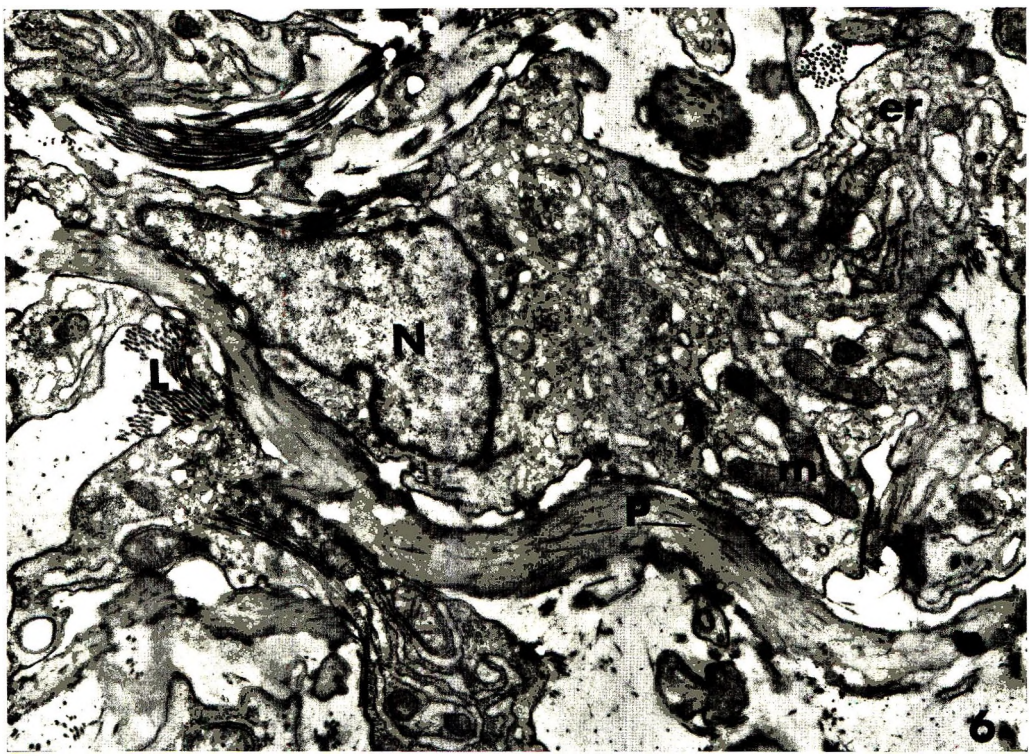

Fig. 6 Tunica media of aorta of control embryo. The cell contains an elongated nucleus (N) mitochondria (m), and rough-surfaced endoplasmic reticulum (er). The elastic fiber has smooth surfaces and consists of many delicate fibrils (P) embedded in a matrix of low electron density. Collagenous fibers (L) are also present in the extracellular space. × 20,000.

bryos were round and had long extensions which were outpouching of the cell membranes. The nuclei of these cells were vesicular, round, bound by smooth membranes, and constituted a large area of the cells. Although rough-surfaced endoplasmic reticulum and mitochondria were observed in these cells, these organelles were fewer in number in cells of deficient than control aortas. No filaments were seen in these cells.

The extracellular spaces in the tunicae media of deficient embryos were more expansive than those of control embryos. This was caused by the lack of organization of cells into layers in the deficient tissue (fig. 7). Elastic fibers in the form of globules, or occasional short strands, were present in the extracellular space, along with collagenous fibers, unidentified fine fibrils, and electron translucent matrix. Rarely, the extracellular space was devoid of elastic fibers. Short strands of elastic fibers had knobby surfaces (fig. 8). The fibers were either closely associated with the cell surface, or they were free in the extracellular space. In the former situation, there was smudging of the cell membrane where elastic fibers were attached, and frequently such fibers were seen in indentations of the cell membrane. Elastic fibers were composed of an electron opaque matrix and fibrils. Such fibrils appeared to be slightly swollen, scattered, and decreased in number, as compared with fibrils in elastic fibers of control embryos. Collagenous fibers appeared to be morphologically normal because they had periodicity.

DISCUSSION

Copper deficiency in laying hens with resulting embryonic death has been produced by others (5), using the procedures

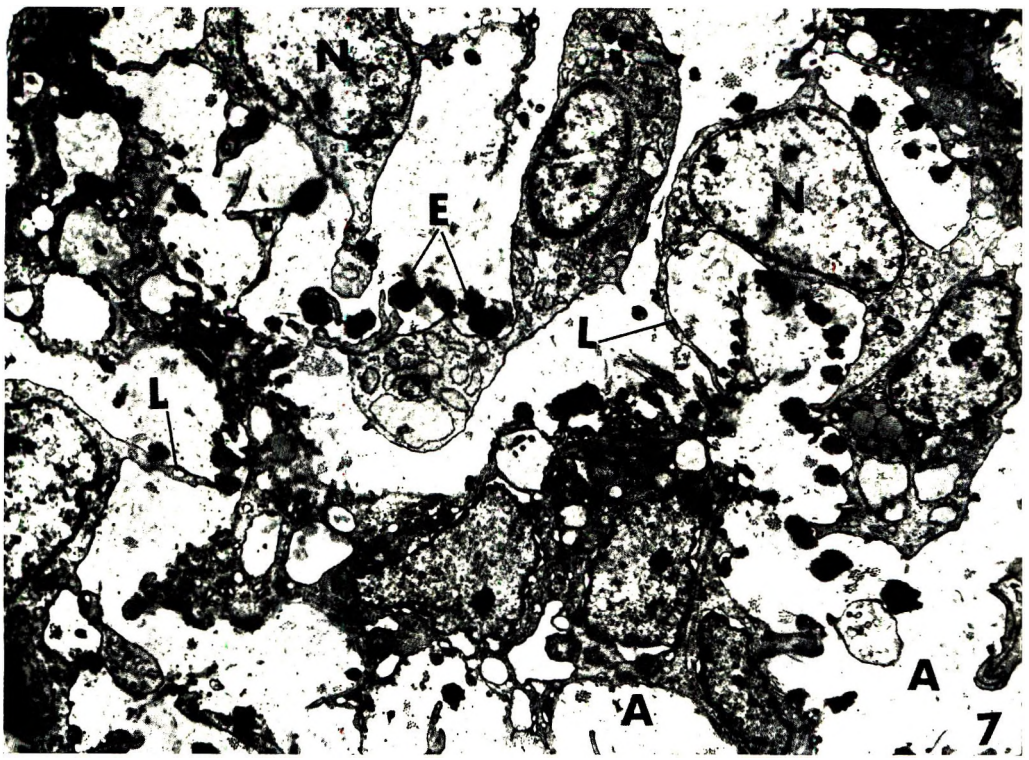

Fig. 7 Tunica media of aorta of copper-deficient embryo. The oval-shaped cells are scattered. They have vesicular nuclei (N) and long extensions (L). Electron opaque elastic fibers (E) are globular. Elastic fibers are absent in some areas (A). × 8,000.

outlined in the present paper. However, they reported that deaths occurred only in the early blood stage, and abnormalities resulting from a copper deficiency were not studied. In the present study, it was shown that the last embryo from hens fed a copper-deficient diet and which survived to 18 days had microscopic lesions of copper deficiency. Thus, by this technique it was possible to study copper deficiency in the developing chick embryo.

It has been shown by chemical analysis that the aorta of the copper-deficient chick accumulates a large fraction of nonelastin, noncollagen protein, and an increased concentration of soluble collagen (13, 14). Isolated elastin contained an elevated concentration of lysine but less desmosine and isodesmosine (15). Chemical analysis of copper-deficient pig aortas showed a decrease in elastin, an increase in hexosamine, but no change in collagen content (16). Thus, it has been suggested that copper is involved in elastin biosynthesis. The data in the present paper appear to confirm this hypothesis by morphologic studies, and also indicate why deficient aortas contain decreased elastin by chemical analysis. The tunicae media of aortas of control 18-day chick embryos contained cells lacking filament which appeared to be fibroblasts or undifferentiated myoblasts, since smooth muscle cells have a uniform cytoplasm, and contain few mitochondria, scant endoplasmic reticulum, and myofilaments. This observation is in agreement with a previous electron microscope study of the normal 18-day chick aorta (17). However, the cells in the tunicae media of 18-day copper-deficient embryos appeared to be mesenchymal cells. Therefore, in copper-deficient embryos there appeared to be retardation of maturation of cells in the tunicae media of aortas and the synthesis by these cells of elastic fibers that differed morphologic-

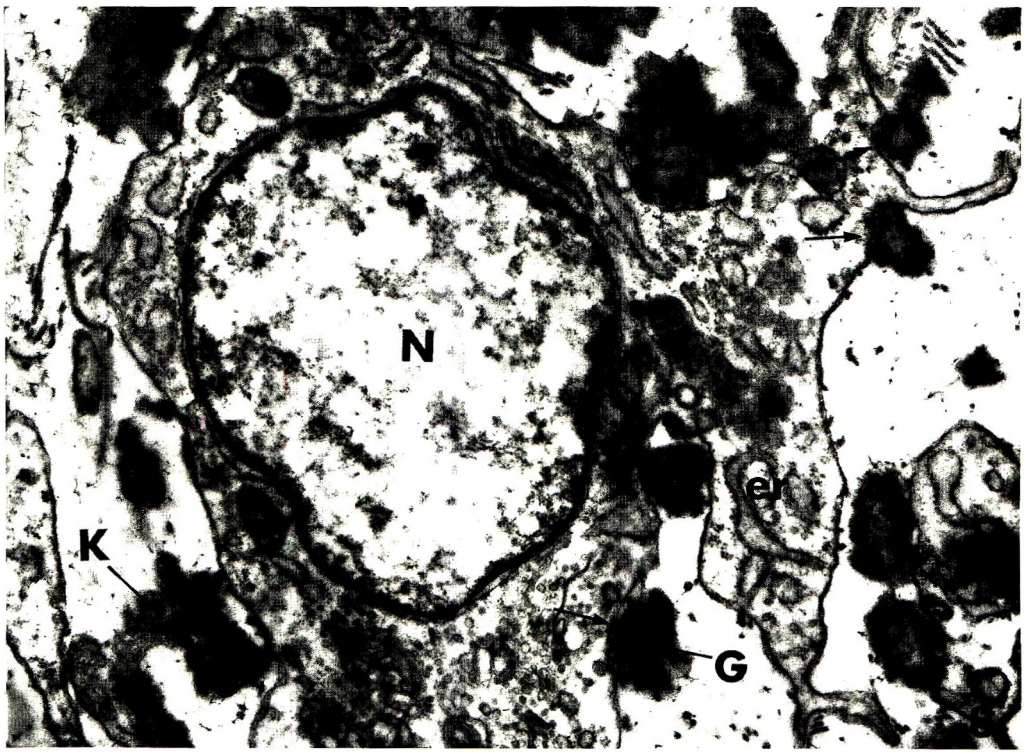

Fig. 8 Tunica media of aorta of a copper-deficient embryo. The cell contains a vesicular nucleus (N), rough-surfaced endoplasmic reticulum (er) and mitochondria. Elastic fibers are globular (G) or short strands with knobby (K) surfaces. They contain a few fibrils embedded in an electron opaque matrix. There is smudging of the cell membranes (arrows) where they contact elastic fibers. × 20,000.

ally from those present in the tunicae media of control aortas of the same age. It has been reported that wet elastin isolated from copper-deficient chick aortas has less elasticity and tensile strength and tends to be more "sticky" than elastin isolated from normal chick aortas (13). The physical properties of connective tissue cannot be determined from thin sections examined by electron microscopy. However, it is probable that since elastic fibers in the aortas of deficient embryos consisted of an electron opaque matrix and a few scattered elastic fibrils, they had less elasticity and a different consistency than elastic fibers in aortas of control chick embryos which consisted of parallel fibrils embedded in a matrix of low electron density.

Lesions of the aortas of copper-deficient chicks observed by the electron microscope have been described previously (3), and were similar to those described in the present paper. The elastic fiber changes were described as degenerative in nature, commencing at the periphery of the fibers. In retrospect, it appears that in the previous work (3) the elastic fiber changes resulted from the synthesis of altered elastin, or the synthesis of altered elastin on the periphery of previously formed normal elastic fibers.

It is not known whether consumption of copper-deficient diets by dams of species other than the chicken cause a similar influence on the integrity of the connective tissue of the offspring and early embryonic death.

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Effect of Chronic Ethanol Feeding on Rat Liver Phospholipid [1,2]

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ABSTRACT Determinations of liver phospholipid concentration and composition were made in experiments to investigate the effect of chronic ethanol ingestion on liver phospholipid metabolism in the rat. Each ethanol-fed animal was grouped with a control animal pair-fed the basal diet plus sucrose and a control animal fed the basal diet ad libitum. Both male and female animals fed ethanol developed increased concentrations of liver phospholipid; the increment in the males was greater than that in the females. Quantitative two-dimensional thin-layer chromatography of the phospholipid fractions showed an increase in lysolecithin and phosphatidyl ethanolamine in the ethanol-fed animals, whether calculated on the basis of phospholipid concentration or total liver content. The increase in lysolecithin, a membrane-lysing agent, may be relevant to alterations reported in experimental and clinical chronic alcoholism.

Evidence obtained in recent studies suggests that ethanol may alter cell membrane permeability and transport (1–7). Since phospholipid plays an important role in the structure and function of cell membranes, for example, membrane permeability and flow of solutes (8), it is possible that the effect of ethanol on membranes is mediated through alteration of membrane phospholipid. Studies in vitro indicate that ethanol can influence phospholipid-protein complexes (8, 9), thus suggesting a mechanism by which ethanol might alter the phospholipid-protein structure of cell membranes. In chronic ethanol poisoning, a change in phospholipid content might be anticipated because the compounds that affect membrane permeability stimulate phospholipid synthesis (10). The present experiments were undertaken to investigate the effect of chronic ethanol ingestion on phospholipid metabolism in the rat. They included determinations of phospholipid concentration and composition.

EXPERIMENTAL

Weanling rats of the Wistar strain were fed a basal diet which included 30% casein and adequate amounts of vitamins together with 30% ethanol (w/v), as described previously (4), for a period of 4 to 15 months. The ingredients of the basal diet are shown in table 1. Ethanol was offered in Richter tubes to minimize evaporation. The basal diet was made into a thick paste with water and was offered fresh, twice weekly, in small jars to avoid spillage. Each ethanol-fed animal (group 1) was paired with a control animal fed the basal diet plus sucrose in isocaloric amounts (group 2) and also with a control animal fed the basal diet ad libitum (group 3). Two experiments were conducted with the animals maintained on these regimens. In the first experiment, the males fed ethanol consumed an average of 4.3 g of ethanol/day and 5.9 g of protein/day. Ethanol accounted for 27% and protein for 21% of the calories/day. Table 2 shows the proportions of ethanol and of protein, carbohydrate, and fat consumption in the diet, and the average weight gain of the animals in the 3 groups in experiments 1 and 2. At the time of liver biopsy, the average weight gain of the ethanol-fed animals was considerably less than that of the isocaloric sucrose pair-fed control animals (table 2). The reason for this phenomenon is not known. Lieber et al. (11) suggest that either alcohol affects food utilization or increases caloric loss, or that the calories from ethanol are less available for growth.

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[2] Presented in part at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1966 (French, S. W. 1966 Chronic ethanol feeding and rat liver phospholipid content. Federation Proc., 25: 478 (abstract)).

TABLE 1

Composition of basal diet

	%
Casein, vitamin-free	30
Sucrose	28
Starch	28
Corn oil	2
Hydrogenated cottonseed oil [1]	6
Non-nutrient fiber	2
Hawk Oser salt mixture [2]	4
	mg/kg
Choline dehydrogen citrate	2,000
Inositol	100
p-Aminobenzoic acid	5
Nicotinic acid	20
Menadione	5
Thiamine·HCl	3
Pyridoxine·HCl	3
Riboflavin	5
Ca pantothenate	25
Folic acid	2
Biotin	0.1
Vitamin B_{12}	0.02
dl-α-Tocopheryl acetate	165
	units/kg
Vitamin D	20,000
Vitamin A	200,000

[1] Crisco, Procter and Gamble Company, Cincinnati.
[2] Hawk, P. B., and B. L. Oser. Science, 74: 369, 1931.

Exp. 1. Phospholipid concentration. In this experiment, 8 male rats were fed ethanol for 7 months, and 8 female rats, for 4 months. When the regimen was terminated, the animals were anesthetized and liver tissue was obtained by biopsy. Lipid was extracted and purified according to the method of Folch et al. (12). Phosphorus was measured by the method of Chen et al. (13).

Exp. 2. Isolation and measurement of phospholipid fractions. Four male rats were fed ethanol for 15 months. In this experiment the animals were killed and whole liver weights were obtained to enable subsequent determinations of the content of each phospholipid fraction.

Purified lipid extract was prepared as in the first experiment. Quantitative two-dimensional thin-layer chromatography (TLC) using the purified lipid extract was used to measure the phospholipid fractions.

Individual phospholipid fractions were isolated by a modification of the method described by Abramson and Blecher (14). Chromatoplates (20 × 20 cm) of 500 μ thickness were prepared with a suspension of 30 g of Silica Gel G in 63 ml of 0.01 M

TABLE 2

Diet and ethanol consumption for animals (males)[1] in experiments 1 and 2

Experiment No.	Duration	Avg daily intake		% of diet								Avg weight gain		
		Ethanol	Food	Ethanol		Protein		Carbohydrate		Fat		Basal + ethanol	Basal + sucrose	Basal ad libitum
				Wt	Cal.	Wt	Cal.	Wt	Cal.	Wt	Cal.			
	months	*ml*	*g*	%	%	%	%	%	%	%	%	*g*	*g*	*g*
1	7	14.35	19.6	18.9	27.0	25.8	21.0	48.4	39.3	6.9	12.7	295	420	496
2	15	17.3	26.4	17.3	24.8	26.4	21.7	49.3	40.5	7.0	13.0	500	668	671

[1] Eight males in experiment 1 and 4 males in experiment 2.

TABLE 3

Effect of chronic ethanol feeding on liver phospholipid concentration

Group	Diet	Phospholipid [1]	
		Male [2]	Female [2]
1	Basal + ethanol	38.2 ± 0.8 [3]	35.8 ± 0.9 [4]
2	Basal + sucrose	30.4 ± 1.1	26.6 ± 1.4
3	Basal ad libitum	30.1 ± 0.7	33.1 ± 0.8

[1] μmoles phospholipid phosphorus/gram wet liver (mean ± SE).
[2] Eight rats/group.
[3] $P < 0.001$ compared with groups 2 and 3.
[4] $P < 0.05$ compared with groups 2 and 3.

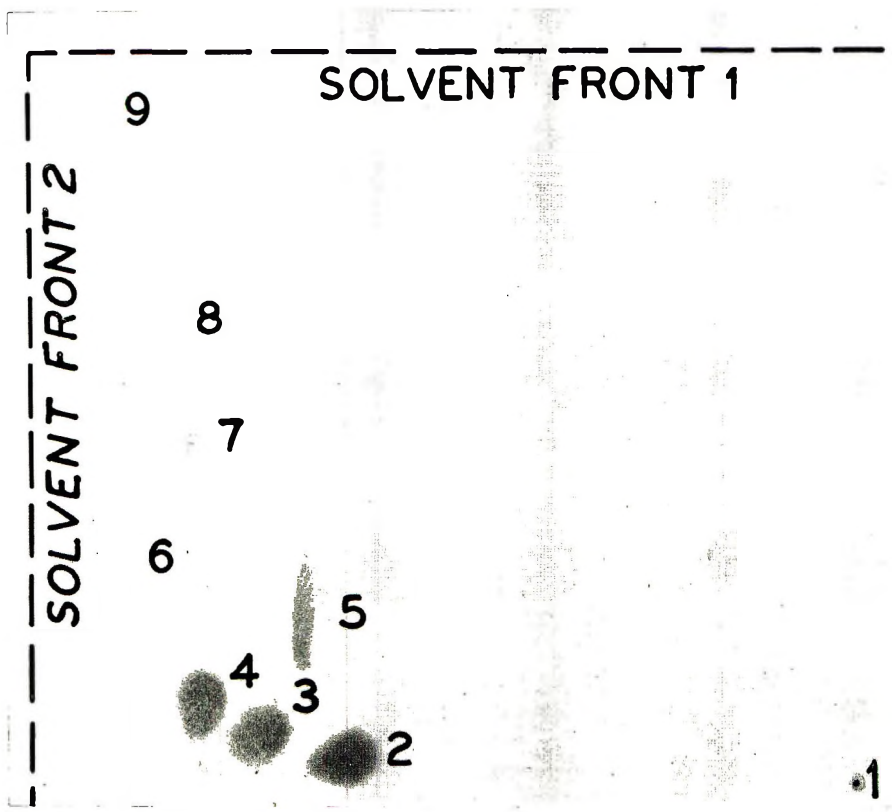

Fig. 1 Separation of a mixture of reference phospholipids (50 μg each) by two-dimensional thin-layer chromatography and visualization of spots with molybdenum spray: 1, origin; 2, lysolecithin; 3, sphingomyelin; 4, phosphatidyl choline; 5, phosphatidyl inositol and serine; 6, unidentified spot seen in the reference phosphatidyl inositol, phosphatidyl choline, and phosphatidyl ethanolamine; 7, phosphatidyl ethanolamine; 8, unidentified spot seen in reference phosphatidyl choline; and 9, cardiolipin.

Na_2CO_3. After activation of the plates at 110° for 30 minutes, samples (50 μliters) were applied as a spot of less than 5-mm diameter on the lower right corner of the plates under a stream of nitrogen. Plates were first developed in a Colab developing chamber with chloroform-methanol-glacial acetic acid-water 250:74:19:3 (v/v). The plates were dried in air for one hour and were then developed in the second dimen-

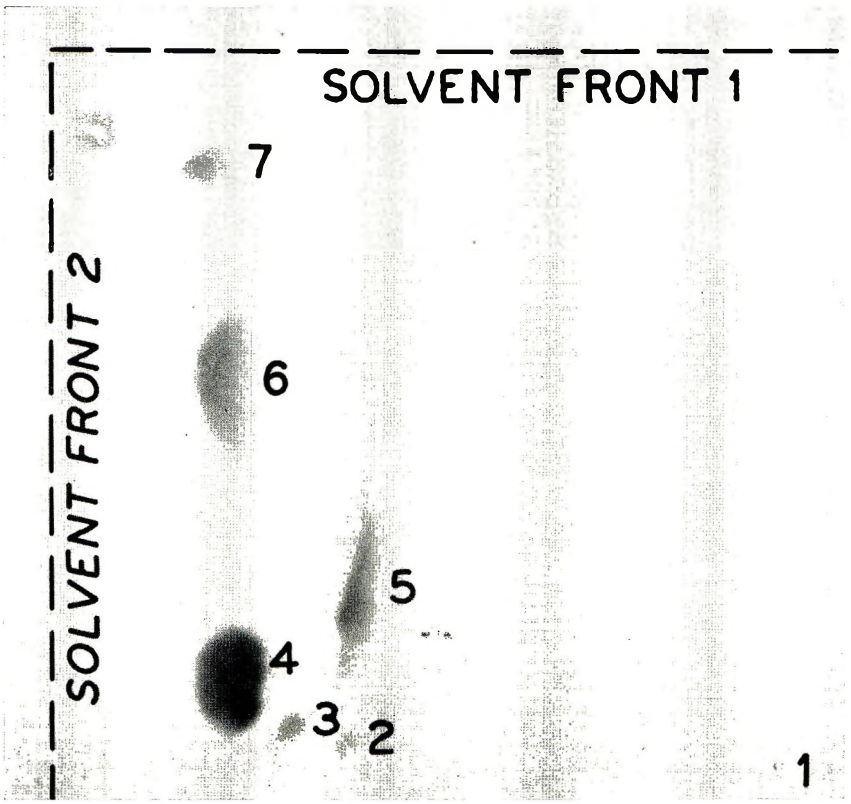

Fig. 2 Two-dimensional thin-layer chromatography of purified lipid extract of rat liver. Spots were identified by comparison with the reference phospholipids: 1, origin; 2, lysolecithin; 3, sphingomyelin; 4, phosphatidyl choline; 5, phosphatidyl inositol and phosphatidyl serine; 6, phosphatidyl ethanolamine; and 7, cardiolipin.

sion (90° rotation clockwise) with chloroform-methanol-7 M ammonium hydroxide 230:90:15 (v/v). Developed plates were dried in air for 15 minutes, sprayed with 0.2% iodine in methanol, and the yellow spots were outlined. The next day the silica gel in each spot was scraped onto glassine weighing paper and then transferred to a 12-ml conical centrifuge tube. Phospholipids were eluted from the silica gel by the method of De Bohner et al. (15). To eliminate the nonspecific effect of the gel in the phosphorus determination, the same extraction procedure was carried out on silica gel from blank portions of comparable size to the phospholipid spots. Quantitative phosphorus analysis of the eluted phospholipid was performed by the method of Marinetti (16).

Identification of individual phospholipid spots was accomplished by comparing their positions with those of reference compounds.[3] Phospholipids were stained with a molybdenum blue reagent (17). In addition, phosphatidyl ethanolamine and phosphatidyl serine were stained with 0.2% ninhydrin in 99% *n*-butanol and 1% pyridine (18).

RESULTS

Exp. 1. Phospholipid concentration. Since the whole liver weight was not obtained in this experiment, no statement can be made regarding total liver phospholipid content. The concentration of liver phospholipid was significantly increased by chronic ethanol feeding in both the

[3] Cardiolipin was obtained from Sylvana Chemical Company, Milburn, New Jersey. The other reference compounds, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine, phosphatidyl enthanolamine, lysolecithin, and sphingomyelin, were obtained from Sigma Chemical Company, St. Louis.

male and female animals. The increment in the males was greater (table 3). This may be owing to the longer period of feeding males (7 months). Lieber et al. (11) observed a difference in phospholipid concentration in male rats fed ethanol and pair-fed isocaloric sucrose-supplemented controls after 10 and 24 days, respectively.

Exp. 2. Phospholipid composition. With the exception of phosphatidyl serine and phosphatidyl inositol (figs. 1 and 2), the individual phospholipid fractions were completely separated by two-dimensional, thin-layer chromatography. Thus the values for these 2 fractions are shown together in table 4, which lists the values for each fraction calculated as total liver content and as concentration. In general, recovery of the phospholipid fractions was good. It was better in the animals fed ethanol and in the controls fed the basal diet ad libitum than in the sucrose-fed animals. The lysolecithin and phosphatidyl ethanolamine fractions were increased in the ethanol-fed animals, compared with both control values, when calculated as total content and as concentration.

DISCUSSION

The increase in liver phospholipid concentration in rats fed ethanol for 4 months or longer reported here is in agreement with the results of Lieber et al. (11), who reported that short-term feeding (10 and 24 days) increased the liver phospholipid concentration and content. These results can be correlated with those of Beard and Barboriak (19), who observed an increase in plasma phospholipid in dogs during chronic ethanol feeding. Seakins and Robinson (20) noted an increase in the rate of synthesis during the recovery phase, 16 hours after a single tube feeding of ethanol. However, the effect of acute ethanol feeding on liver phospholipid may well be different from that of chronic ethanol feeding: various investigators (20–23) have reported that the concentration of liver phospholipid is not increased after acute ethanol feeding, and Scheig and Isselbacher (24) have shown that the incorporation of fatty acids into triglyceride is stimulated at the expense of phospholipid.

TABLE 4

Thin-layer chromatography of liver phospholipid: concentration and total content of fractions

Phospholipid fractions	Concentration [1]			Total content [2]		
	Diet			Diet		
	Basal + ethanol	Basal + sucrose	Basal ad libitum	Basal + ethanol	Basal + sucrose	Basal ad libitum
Origin	0.30 ± 0.16	0.14 ± 0.11	0.35 ± 0.17	5.2 ± 3.0	3.3 ± 1.9	6.6 ± 2.1
Lysolecithin	0.84 ± 0.02 [3]	0.14 ± 0.07	0.29 ± 0.02	14.0 ± 1.2 [4]	3.6 ± 1.3	5.8 ± 0.5
Sphingomyelin	2.08 ± 0.55	0.81 ± 0.12	1.71 ± 0.39	32.4 ± 5.9	18.7 ± 2.9	26.4 ± 5.2
Phosphatidyl choline	16.96 ± 1.09	11.90 ± 1.54	15.36 ± 1.58	278.7 ± 20.8	274.3 ± 36.4	306.0 ± 36.6
Phosphatidyl inositol and serine	3.69 ± 0.19	2.36 ± 0.19	3.36 ± 2.50	60.6 ± 4.2	44.5 ± 6.0	66.0 ± 1.7
Phosphatidyl ethanolamine	8.52 ± 0.20 [4]	4.46 ± 0.48	5.18 ± 0.36	139.5 ± 6.9 [5]	102.0 ± 10.1	102.8 ± 7.2
Cardiolipin	1.85 ± 0.50	1.21 ± 0.12	1.57 ± 0.17	31.0 ± 8.7	27.9 ± 3.0	31.8 ± 5.1
Total phospholipid recovered	34.30 ± 1.53	20.68 ± 1.24	27.40 ± 1.83	563.0 ± 36.0	475.0 ± 33.0	545.0 ± 49.0

[1] μmoles phospholipid phosphorus/gram wet liver (mean ± SE), 4 male rats/group.
[2] μmoles phospholipid phosphorus/total liver (mean ± SE), 4 males rats/group.
[3] $P < 0.001$ compared with groups 2 and 3.
[4] $P < 0.01$ compared with groups 2 and 3.
[5] $P < 0.05$ compared with groups 2 and 3.

The effect of chronic ethanol ingestion on liver phospholipid is quite different from that of various dietary deficiencies. For example, in deficiencies of choline (25–27), protein (9, 29), pyridoxine (30), or thiamine (31), the liver phospholipid is diminished. It is unlikely, therefore, that ethanol ingestion affects phospholipid metabolism by means of increasing the requirement for essential nutrients.

In the present experiments, chronic ethanol feeding either increased or left unchanged the various liver phospholipid fractions, and none of the fractions were decreased. This is not true in choline deficiency with or without protein deficiency (25, 32), where the lecithin fraction is depressed.

Worthy of note is the previously unreported ethanol-induced increase in lysolecithin. Studies in vitro have demonstrated an increase in lysolecithin in aging rat liver mitochondria, probably as a result of endogenous phospholipase A hydrolysis of phosphatidyl choline (33, 34). Lysolecithin is a membrane-lysing agent (35, 36). Elsbach et al.[4] reported evidence that lysolecithin plays a role in the lysis and renewal of cellular membranes. Feldberg et al. (37) observed that lysolecithin perfused through the dog liver liberated histamine, protein, and pigments, possibly because of its cytolytic action. Nygard et al. (38) showed that lysolecithin in vitro altered the morphology of mitochondria, and Pesch and Peterson (39) produced lysis of mitochondria in vitro with a high concentration of lysolecithin. However, it has not been determined whether the small increase in lysolecithin observed in the studies reported here could account for the membrane structural alterations (40) or functional alterations (1, 5, 4) described in chronic ethanol feeding.

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Effect of Indigestible Oils on Vitamin K Deficiency in the Rat [1,2]

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ABSTRACT Studies of indigestible oils such as mineral oil and squalene were undertaken to determine their effect on vitamin K deficiency in the rat. Severe hypoprothrombinemia occurred in male rats fed a vitamin K-deficient diet containing as little as 0.5% of the oil and the effect was reversed by feeding vitamin K. Female rats were partially resistant to the treatment. Dietary squalene also interrupted the assimilation of vitamin K in chicks where 0.5% of dietary oil inhibited the utilization of 0.1 μg of phylloquinone/g of diet. Similar results were obtained in chicks fed retinyl acetate and retinoic acid.

During studies on nutritional factors influencing the development of vitamin K deficiency in the rat we observed severe hypoprothrombinemia after feeding diets containing squalene or oxidized squalene. Dietary oils have been studied with reference to the assimilation of vitamin and provitamins A, but little evidence is available concerning their effect on the absorption of vitamin K. Elliott et al. (1), Barnes (2) and Javert and Macri (3) have presented evidence that mineral oil by mouth may induce vitamin K deficiency and Nightingale et al. (4) obtained similar results with a triglyceride containing dihydroxystearic acid. By comparison with these, the effect of squalene was unique since it was operative at low dietary concentration and because oxidation of squalene enhanced its effectiveness. These observations led us to study the anticoagulant effect of several indigestible oils, the possible effect of oxidation of dietary lipids on blood coagulation and the use of these oils in the development of vitamin K deficiency in the rat.

METHODS

Experimental details of animal care, diet preparations, blood sampling, and assay of prothrombin have been reported previously (5, 6). With the one exception noted, adult rats of the St. Louis University strain were used. Diets were fed to individually caged animals and prothrombin was measured in cardiac blood, using Russell's viper venom according to Hjort et al. (7). The average concentration of prothrombin and its standard error are given as criteria of the effect of the diet. The reference standard for these assays was a pool of plasma prepared from adult male rats fed commercial laboratory ration.[4]

Oxidation of the various lipids was effected with monoperphthalic acid (8) in ether. After addition of a measured amount of oxidizing reagent, the reaction mixture stood at room temperature until analyses (9) indicated little or no residual peroxide. The initial stage of oxidation of squalene [5] was carried out in an ice bath. Other lipids were oxidized less rapidly and did not require cooling. After oxidation the ether solutions were extracted with sodium carbonate, washed with water and dried over sodium sulfate. The solvent was removed under reduced pressure and the oil was finally dried in vacuo at room temperature.

A standard diet deficient in vitamin K with the following composition was used throughout these studies: casein, 21; corn-

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[2] A preliminary report of some of the studies contained in this paper was presented at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April, 1961.

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[4] Purina Laboratory Chow, The Ralston Purina Company, St. Louis.

[5] Eastman Chemical Company, white label grade; oxidized with 0.167 mole of perphthalic acid per mole of squalene.

starch, 43; glucose monohydrate,[6] 27; corn oil, 5; and a supply of vitamins and minerals as described previously (5).

RESULTS AND DISCUSSION

After feeding the standard deficient diet containing 0.5% squalene or oxidized squalene, low prothrombin levels were observed in both adult and weanling rats (table 1). Although oxidized squalene appeared to be more effective, the feeding of either oil resulted in depressed coagulation activity. As shown in table 1, the anticoagulant effect was countered by the administration of vitamin K.

Table 2 shows the effect of these oils fed to female rats of the same age and strain. As observed earlier (5), prothrombin levels in the female were higher than in the male. Furthermore, female rats exhibited little depression of prothrombin due to ingestion of squalene. This may be related to the general resistance of female rats to vitamin K deficiency (10). Previously we observed that female rats are similarly insensitive to the hemorrhagenic toxicity of vitamin A (5).

TABLE 1

Prothrombin levels in male rats fed vitamin K-deficient diets containing 0.5% of squalene and oxidized squalene

Dietary additions	Prothrombin	
	Weanling [1]	Adult [2]
	% of normal	*% of normal*
None	74 ± 7 [3] (10)	75 ± 5 (20)
Squalene	19 ± 5 (9)	24 ± 5 (10)
Oxidized squalene	7 ± 3 (6)	10 ± 1 (10)
Oxidized squalene + vitamin K [4]		101 ± 3 (10)

[1] Weanling male rats fed the experimental diet for 8 weeks. Number of animals is shown in parentheses.
[2] Adult male rats fed the experimental diet for 2 weeks.
[3] SE.
[4] 2.5 μg of phylloquinol diphosphate/g of diet.

TABLE 2

Prothrombin levels in adult female rats fed squalene and oxidized squalene

Dietary additions	Prothrombin
	% of normal
None	136 ± 3 [1] (9) [2]
0.5% squalene	106 ± 5 (9)
0.5% oxidized squalene	79 ± 12 (10)

[1] SE.
[2] Number of animals is shown in parentheses.

In each of these experiments, oxidized squalene appeared to be more effective than the untreated oil and the possibility existed that untreated squalene was contaminated with products of air oxidation. Squalene was purified by molecular distillation [7] and by formation of the thiourea clathrate (11). Diets containing purified squalene were also protected with an antioxidant. The results of these studies shown in table 3 provide no evidence that oxidation products are required for the anticoagulant effect. Furthermore, oxidized squalene fed at 0.05% of the diet (imitating the possible presence of 10% oxidized impurity in untreated squalene) had no effect on prothrombin concentration.

TABLE 3

Prothrombin levels in rats fed 0.5% purified squalene

Purification	Prothrombin
	% of normal
Molecular distillation	19 ± 2 [1] (20) [2]
Thiourea adduct	18 ± 2 (19)
Molecular distillation + antioxidant [3]	23 ± 2 (39)

[1] SE.
[2] Number of animals is shown in parentheses.
[3] 0.05% ethoxyquin (Santoquin, Monsanto Company, St. Louis).

Despite this evidence that oxidation was not a prerequisite for the anticoagulant effect of fed squalene, we examined several oxidized oils for possible induction of vitamin K deficiency. Cottonseed oil, beef fat and cod liver oil were oxidized with 0.6 mmole of perphthalic acid per gram; methyl oleate and methyl linoleate were oxidized with one double bond-equivalent of perphthalic acid; and oxidized soybean oil was obtained as a commercial product.[8] Each of these oils mixed into the standard vitamin K-deficient diet replacing corn oil was fed to adult male rats without resulting in the symptoms of anticoagulation noted after feeding squalene. Thus oxidation of edible oils does not appear to make them effective in the induction of vitamin K deficiency.

On the basis of these data it appeared that the effects of squalene and oxidized

[6] Cerelose, Corn Products Company, New York.
[7] CMS-5 centrifugal molecular still, Consolidated Vacuum Corporation, Rochester, New York.
[8] Drapex 6.8, Argus Chemical Company, Brooklyn, New York.

squalene were related to their indigestibility and that other oils may be equally effective. Table 4 contains data obtained with squalane, mineral oil and other lipids fed at 0.5% of the diet. Squalane and mineral oil were as effective as squalene. Castor oil did not lower prothrombin and hence the effect observed with other oils is probaly not due to increased laxation. Dihydroxystearic acid at this low level also did not affect prothrombin concentrations; however, Lockhart et al. (4) have observed previously that rats fed approximately 8% of this acid in glyceride form had prolonged prothrombin times and became hemorrhagic. As observed with squalene, the anticoagulant effect of mineral oil was countered by the administration of vitamin K.

Although squalene may occur in foods, the concentration of this oil is probably never sufficient to influence prothrombin levels. Rothblat et al. (12) reported 40 to 50 μg of squalene per g of rat liver which is approximately 0.1% of rat liver lipid. We have assayed several other lipids by the same procedure and observed similar low levels of squalene. The following percentages were detected: beef fat, 0.05; corn oil, 0.06; soybean oil, 0.15; and cottonseed oil, 0.03. These data and those obtained with oxidized oils indicate clearly that dietary anticoagulation due to lipids does not occur unless by the deliberate administration or ingestion of effective amounts of oil.

In mammalian species, simple nutritional deficiency of vitamin K in intact animals leads to moderate hypoprothrombinemia so that the anticoagulant effect of dietary supplements can be clearly superimposed on the effects of deficient regimen; however in chicks, simple deficiency results in severe hypocoagulation which cannot be significantly intensified by further treatment. In chicks the effects of squalene were observed if the oil was fed in the presence of vitamin K. The results are shown in table 5. Under the conditions fed, 0.5% squalene lowered prothrombin in the chick in a manner equivalent to the loss of 0.1 μg of vitamin K/g of diet. Similar interference with the utilization of vitamin K in chicks was also observed after feeding diets containing 2500 IU of retinyl acetate and 250 IU of retinoic acid/g of diet. In these experiments prothrombin levels indicated that 0.1 to 0.15 μg of vitamin K/g of diet was neutralized or rendered otherwise unavailable. Previous studies by Quick and Stefanini (14) indicated that excessive doses of vitamin A in the chick had no effect on one-stage prothrombin times. Differences in our experimental design preclude a clear interpretation of this difference, but the significance of the data in table 5 clearly indicates that interrupted utilization of vitamin K by indigestible oils and vitamin A previously observed only in mammals also occurs in the chick.

On the basis of the data presented here, indigestible oils at relatively low concen-

TABLE 4

Prothrombin levels in rats fed 0.5% of various oils

Dietary additions	Prothrombin
	% of normal
None	75±5 [1](20) [2]
Squalane	25±3 (10)
Mineral oil	19±3 (10)
Castor oil	68±5 (10)
DHSA [3]	66±7 (10)
Mineral oil + vitamin K [4]	100±2 (10)

[1] SE.
[2] Number of animals is shown in parentheses.
[3] Dihydroxystearic acid.
[4] 2.5 μg of phylloquinol diphosphate/g of diet.

TABLE 5

Prothrombin levels in chicks fed diets containing squalene, retinyl acetate or retinoic acid

Dietary additions [1]	Prothrombin [2]
	% of normal
None	7±1 [3](18) [4]
0.05 μg vitamin K [5]	17±1 (16)
0.1 μg vitamin K	33±1 (17)
0.2 μg vitamin K	64±4 (18)
0.2 μg vitamin K + squalene [6]	36±7 (18)
0.2 μg vitamin K + retinyl acetate [7]	21±2 (18)
0.2 μg vitamin K + retinoic acid [8]	28±3 (16)

[1] Day-old chicks fed for 2 weeks diets containing the additions shown; for the composition of the vitamin K-deficient diet for chicks, see Matschiner and Doisy (13).
[2] Conditions of the prothrombin assay have been described previously (13).
[3] SE.
[4] Number of animals is shown in parentheses.
[5] Additions of phylloquinone in μg/g of diet.
[6] 0.5% squalene.
[7] 2500 IU retinyl acetate/g of diet.
[8] 250 IU retinoic acid/g of diet.

tration in the diet profoundly enhance vitamin K deficiency in the rat. Apparently, the massive amounts of mineral oil used earlier by Elliott et al. (1) and others are not necessary to produce the deficiency if proper dietary control is maintained. In addition, the present data substantiate that several oils may replace mineral oil in its effect on the assimilation of vitamin K. In an early study, Mitchell (15) observed that higher melting paraffins were not as effective as liquid paraffins in restricting the absorption of provitamins A from spinach, and Bacon et al. (16) made reference to the production of vitamin K deficiency in rats by feeding polymerized sardine oil. The present study extends this list of effective oils to squalene, squalane and oxidized squalene and suggests that indigestible oils may be convenient agents for assistance in the development of vitamin K deficiency in mammalian species.

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Mechanism of the Effect of Retinoic Acid and Squalene on Vitamin K Deficiency in the Rat [1]

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ABSTRACT The anticoagulant effect of squalene and retinoic acid was studied in adult male rats fed purified diets deficient in vitamin K. Both appear to inhibit absorption of vitamin K although possibly by different mechanisms. The requirement for vitamin K increased with a dietary increase of squalene but maximal action of retinoic acid was easily obtained and further ingestion of vitamin A did not affect prothrombin levels. The requirement for vitamin K in rats fed 0.5% squalene or 50 IU of retinoic acid/g of diet was about 9 μg/day which resembles the requirement in germfree rats. On this basis indigestible oils and retinoic acid may be useful agents for the production of "simple" vitamin K-deficiency in mammalian species.

An effect of dietary lipids on vitamin K deficiency has been observed with a number of compounds ranging in composition from indigestible oils such as mineral oil and squalene, through substances of questionable dietary significance such as dihydroxystearic acid, tocopherolquinone and decanal, to the physiologically important members of the vitamin A family — the retinyl esters and retinoic acid (1). The mechanism of action of these compounds with respect to vitamin K deficiency is not known, but a number of speculations have been made. Mineral oil may affect the absorption of vitamin K; tocopherolquinone was considered to act systemically as an inhibitory structural analogue of vitamin K; and vitamin A has been viewed both as a systemic and an intestinal anticoagulant (2, 3).

Of these lipids, retinoic acid and squalene seemed most worthwhile for further studies. Squalene stimulates vitamin K deficiency in a dependable and effective way. Like mineral oil and other indigestible hydrocarbons, it probably interrupts the absorption of vitamin K. Retinoic acid is a remarkably potent antagonist to vitamin K in the rat and offers an excellent opportunity to examine the mechanism of hemorrhagenic toxicity of vitamin A.

METHODS

Experimental details of animal care, diet preparation, blood sampling and assay of prothrombin have been reported previously (4, 5). Diets were fed for 2 weeks to individually caged adult male rats of the St. Louis University strain. Prothrombin was measured in cardiac blood according to Hjort et al. (6). The average concentration of prothrombin and its standard error are given as criteria of the effect of the diet. The reference standard for these assays was a pool of plasma prepared from adult male rats fed commercial laboratory ration.[2] As determined earlier, single values less than 85% were considered deficient.

The vitamin K-deficient diet used throughout these studies had the following composition: casein, 21; corn starch, 43; glucose monohydrate,[3] 27; corn oil, 5; and a supplement of vitamins and minerals as described previously (4).

RESULTS AND DISCUSSION

The most direct evidence of the site of action of both squalene and retinoic acid was obtained by injection of these substances into vitamin K-deficient rats. The results in table 1 indicate clearly that neither retinyl acetate nor retinoic acid are effective anticoagulants after intracardial injection. Sufficient vitamin A was injected to produce severe hypoprothrom-

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

[3] Cerelose, Corn Products Company, New York.

TABLE 1

Effect of method of administration on the anticoagulant properties of squalene and vitamin A

Experiments with squalene			Experiments with vitamin A		
Treatment [1]	Prothrombin	Normal values [2]	Treatment [1]	Prothrombin	Normal values [2]
	% of normal			*% of normal*	
None	81 ± 3 [3]	19/40	Ester (ic)	67 ± 5	3/17
0.1 ml/3 day (ic)	79 ± 8	6/18	Vehicle (ic)	79 ± 6	8/19
			Ester (ip)	84 ± 6	10/20
0.1 ml/2 day (ip)	87 ± 4	11/20	Vehicle (ip)	76 ± 4	7/19
0.1 ml/2 day (oral)	67 ± 6	7/19	Acid (ic)	82 ± 3	6/19
			Vehicle (ic)	78 ± 3	7/18
0.1 ml/day (oral)	46 ± 5	0/19	Acid (oral)	11 ± 2	0/10
			Ester (oral)	23 ± 8	0/10

[1] Squalene was injected intracardially (ic) or intraperitoneally (ip) in the amounts and at the intervals shown; 15,000 IU of retinyl acetate (ester) were injected at 3-day intervals in 0.1 ml of 5% polyoxyethylene sorbitan monooleate (Tween 80) in saline which was the vehicle; 1500 IU of retinoic acid were injected at 3-day intervals as the sodium salt in saline which was the vehicle. Retinoic acid was fed (oral) at 50 IU/g of diet; retinyl acetate was fed (oral) at 500 IU/g of diet.
[2] Number of prothrombin concentrations greater than 85%/total observations.
[3] SE.

binemia if fed in a vitamin K-deficient diet. The results of feeding vitamin A are shown in table 1 for comparison. In a later experiment, vitamin K-deficient rats were injected intracardially with as much as 6000 IU of retinoic acid at 3-day intervals without significant depression of prothrombin.

Results with squalene were similar. The concentration of prothrombin was unchanged after intracardial or intraperitoneal administration of the oil. In these experiments the amount of squalene which could be injected intracardially was less than the effective dose administered by mouth, and hence it became necessary to estimate possible absorption of dietary squalene. The feces of rats fed the purified diet with or without added squalene were collected daily and analyzed to determine recovery of the oil. Squalene was purified and measured according to Rothblat et al. (7). The purified oil was also identified by gas chromatography. The results of these studies are shown in table 2. Approximately 53% of the ingested squalene was recovered in the feces. An additional amount of excess fecal lipid equivalent to the remaining fed squalene was recovered but the chromatographic properties of this lipid were indistinguishable from those observed with lipid from control feces. These results indicate that some squalene was transformed during ingestion, digestion and recovery, but together with the data obtained in the injection studies they substantiate that the anticoagulant effect of dietary squalene does not depend on absorption of the ingested oil.

Thus squalene and retinoic acid exert their anticoagulant effects in the intestine by interrupting in some way the supply of vitamin K. But the mechanism is obscure. Recently we made a study of fecal elimination of vitamin K to ascertain whether any changes occur in absorption or synthesis of vitamin K. Feces were collected daily, stored at −5° and assayed for vitamin K in the chick (8). As shown in table 3, rats fed the basal deficient diet for 2 weeks excreted 1415 μg of vitamin K. Feces from rats fed the diet containing 0.5% squalene contained 1095 μg. If vitamin K was added with squalene so that the rats ingested about 700 μg of vitamin

TABLE 2

Fecal lipids from rats fed purified diets with and without added squalene [1]

	Without squalene	With squalene [2]
Fecal lipid (total), g	8.1	20.8
Fecal squalene, g	0.02	7.4

[1] The total feces of 130 rat days were examined in each experiment.
[2] Approximately 14 g of squalene were ingested.

TABLE 3

Fecal vitamin K in adult male rats

Dietary additions [1]	Feces [2]	Vitamin K				
		Fecal [3]		Eaten	Recovered	
	g	μg/g	μg	μg	μg	%
None	101	14.0	1415			
Squalene	115	9.5	1095			
Squalene + vitamin K	114	14.5	1655	700	560	80
Vitamin K	100	15.7	1570	600	155	25
Retinoic acid	107	12.7	1360			
Retinoic acid + vitamin K	98	17.0	1665	600	305	51

[1] The following additions were made where indicated: squalene, 0.5%; phylloquinone (vitamin K), 0.25 μg/g of diet; and retinoic acid, 50 IU/g of diet.
[2] Total feces (dry weight, g) were collected from 10 rats for 13 days.
[3] Fecal vitamin K was determined by bioassay and expressed as phylloquinone.

K during the experiment, 560 μg were returned in the feces; but if a similar amount of vitamin K was fed in the control diet only 155 μg were recovered. These data support a mechanism of interrupted absorption and possible diminished bacterial synthesis of vitamin K for the action of dietary squalene.

Retinoic acid did not alter excretion of vitamin K significantly compared with the control diet but gave evidence of impaired absorption. About half of the vitamin K ingested with retinoic acid was recovered in the feces.

These data on fecal excretion of vitamin K were supported by the results of preliminary experiments with radioactive vitamin K. A sample of phylloquinone-3′-methyl-^{14}C (sp. act. 0.1 mCi/mmole)[4] was mixed with the standard deficient diet at a concentration of 0.1 μg/g of diet. The radioactive diet was fed with and without added squalene or retinoic acid. Biological response to the fed vitamin was determined by prothrombin activity and absorption of vitamin K was estimated by measuring fecal radioactivity. The results of these experiments are shown in table 4. Low prothrombin levels were accompanied by greater recoveries of ^{14}C in the feces. Furthermore, chromatographic separation of the fecal radioactivity on silicic acid showed that the phylloquinone fractions obtained from animals fed squalene or retinoic acid contained twice as much ^{14}C as the same fractions obtained from control animals. The livers of the control rats contained 0.5% of the fed ^{14}C but no radioactivity could be detected in the livers of the hypoprothrombinemic rats. The isotopic content of the administered vitamin was too low to permit further exploration of the absorptive and metabolic events which occurred but these data clearly support the view that squalene and retinoic acid inhibit the absorption of fed vitamin K.

TABLE 4

Prothrombin and fecal excretion of ^{14}C in rats fed radioactive vitamin K

Dietary addition [1]	Prothrombin	Fecal ^{14}C [2]	Fecal vitamin K-^{14}C [2]
	% of normal	%	%
None	100 ± 3 [3]	48	30
Squalene	46 ± 7	76	60
Retinoic acid	20 ± 2	73	58

[1] All diets contained 0.1 μg of radioactive phylloquinone/g of diet as described in the text. The following additions were made where indicated: squalene, 0.5%; and retinoic acid, 50 IU/g of diet.
[2] Percentage of ingested radioactivity. Fecal ^{14}C was measured in a total lipid extract of the dried feces. Fecal vitamin K-^{14}C was determined after chromatography of fecal lipids on silicic columns (Mallinckrodt, SilicAR 100–200 mesh). Vitamin K-^{14}C was measured in those fractions which contained authentic samples of phylloquinone.
[3] SE.

Squalene and retinoic acid probably do not interrupt absorption by the same mechanism. Karvinen et al. (9) proposed that most of the increased excretion of cholesterol in rats fed mineral oil was due to solubility of the sterol in the oil. Squalene may have a similar effect on vitamin K but retinoic acid was fed in amounts too small to permit such a mechanism. Evidence of a different site of

[4] This compound was prepared by Michael and Elliott (Federation Proc., 20: 451, 1961) and donated for this study.

TABLE 5

Prothrombin response to dietary vitamin K in rats fed squalene and retinoic acid

Phylloquinone added [1]	Prothrombin			
	Experiments with squalene [2]		Experiments with retinoic acid [3]	
	0.5%	1.0%	50 IU/g	100 IU/g
μg/g	% of normal		% of normal	
—	14±2 [4]		13±3	
0.125	28±4		41±9	57±9
0.250	66±5	11±1	80±6	69±6
0.500	95±4		96±3	102±8

[1] Phylloquinone added to the standard vitamin K-deficient diet.
[2] 0.5 or 1.0% dietary squalene added as indicated.
[3] 50 or 100 IU of retinoic acid added per g of diet as indicated.
[4] SE.

action for retinoic acid was obtained during a study of its quantitative relationship with vitamin K. In contrast with the effect of squalene which depends on the amount of oil ingested, the site of action of retinoic acid was easily saturated. As shown in table 5, doubling the dietary level of retinoic acid from 50 to 100 IU/g of diet did not significantly decrease prothrombin in rats fed three different levels of vitamin K. These data suggest that retinoic acid inhibits only part of the absorptive capacity for vitamin K and that further ingestion of vitamin A is without effect on prothrombin concentration. This explains in part the general absence of hypocoagulation as a symptom of vitamin A intoxication in man. The amount of natural vitamin K normally ingested is probably protective and hence other symptoms of vitamin A intoxication, but not hemorrhage, are observed. By comparison, excessive amounts of mineral oil result in prolonged prothrombin times (10) and require a supplement of vitamin K.

On the basis of the results in table 5, squalene and retinoic acid should be useful for the development of vitamin K deficiency in the rat and other mammalian species. The requirement for phylloquinone in rats fed these compounds is about 9 μg/day (based on 15–20 g of daily food consumption) which is similar to the requirement observed in germfree rats (11, 12). If squalene and retinoic acid are accepted as otherwise innocuous agents, they will be valuable additives for vitamin K-deficient diets in mammalian studies.

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An Evaluation of Factors Affecting Survival of Choline-deficient Weanling Rats with Special Emphasis on Dietary Sodium [1]

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ABSTRACT Extensive death losses due to hemorrhagic kidney damage occur in weanling rats after they have been fed a choline-deficient diet for 7 to 10 days. We have investigated the effect of dietary factors other than choline content, and changes in several physiological functions on the survival of choline-deficient, male, weanling rats during this critical period. Special emphasis was given in these studies to alterations in the sodium content of the diets. A choline-deficient diet was supplemented with organic and inorganic salts, chelating agents, and diuretics. A sodium-deficient, choline-deficient diet was also studied. Feed intake, growth rate, urine volume, urinary mineral excretion patterns and plasma NPN levels were determined. Weanling rats receiving a choline-deficient diet were subjected to a physiological crisis on about the seventh experimental day caused by renal damage. Sixty-two per cent of rats with an initial weight above 75 g survived this crisis, whereas only 46% of the rats with an initial weight less than 75 g survived. The addition of 0.7% NaCl to a choline-deficient diet that contained adequate sodium for growth increased the survival of the heavier rats to 88%. Results of this diet with the lighter rats were variable. The NaCl diet was diuretic and caused an increase in the urinary excretion of Na, Ca and K. The addition of EDTA to the choline-deficient diet increased survival of the heavier rats to 75% and the lighter rats to 81%. EDTA depressed feed intake, urine volume, growth, and delayed the onset of high plasma NPN levels and kidney damage past 7 days. The sodium-deficient, choline-deficient diet also increased survival to 90%, apparently by depressing growth during the first 5 experimental days. None of the diets studied, except the choline-supplemented diet, completely protected rats from hemorrhagic kidney damage.

Various aspects of choline deficiency have been under study in this laboratory for a number of years (see for example (1–4)). Recently we have attempted to obtain new information relating to the often-observed hemorrhagic kidney syndrome that develops in weanling rats after they have been fed a choline-deficient diet for 7 to 10 days. The possibility that the mineral composition of the diet may affect this condition was raised by the report of Sutherland and Hartroft [3] that the addition of 0.7% NaCl to a choline-deficient, sodium-deficient diet protected rats from death or hemorrhagic kidney damage. We have studied the effect of changes in the mineral composition of a choline-deficient diet on survival, kidney damage, plasma nonprotein nitrogen (NPN) levels, growth, feed intake, urine volume, and renal excretion of several minerals in rats.

EXPERIMENTAL PROCEDURE

The formula of the basal choline-deficient diet used in these studies is given in table 1. Other diets were obtained by varying the mineral composition or adding the disodium salt of ethylenediaminetetraacetic acid (EDTA) or choline chloride to the basal diet. Additions to the diet were made at the expense of sucrose. These diets were relatively high in fat. They were also low in methionine and vitamin B_{12} to limit the *de novo* synthesis of choline. The peanut meal and casein used in the diets were extracted 48 hours with 82% meth-

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[2] Dr. W. D. Salmon, Professor Emeritus, died February 5, 1966.
[3] Sutherland, L., and W. S. Hartroft 1964 A renoprotective effect of sodium supplements in low-choline diets. Federation Proc., 23: 880 (abstract).

TABLE 1
Basal choline-deficient diet

	%
Extracted casein	6
Extracted peanut meal	25
Sucrose	42
Lard	20
Vitamin mix [1,2]	2
Salt mix [3]	5

[1] The vitamin mix furnished the following concentration of vitamins per kilogram of diet: (in milligrams) menadione, 5; riboflavin, 2; thiamine·HCl, 4; pyridoxine·HCl, 2; Ca pantothenate, 10; niacin, 25; inositol, 40; *d*-α-tocopheryl acetate, 55; and vitamin A, 50,000 IU.

[2] Two milligrams of folacin and 1600 USP units of calciferol per kilogram of diet were added in addition to the vitamin mix.

[3] The salt mix furnished the following minerals per kilogram of diet: (in g) Ca phosphate, 31.50; potassium chloride, 6.00; magnesium sulfate, 4.00; sodium bicarbonate, 7.00; ferric citrate, 1.23; manganous sulfate, 0.12; zinc carbonate, 0.10; cupric sulfate, 0.02; and potassium iodide, 0.03.

anol and 57 hours with 100% methanol in a continuous-extraction apparatus to remove choline-containing phospholipids.

Charles River CD strain male weanling rats weighing approximately 45 to 75 g were used in most of the experiments. Older rats were used in some of the survival studies. The rats were housed in individual wire-bottom cages in an air conditioned room. The animals had continuous access to the experimental diets and fresh water.

In the first series of experiments over 800 rats in groups of five were fed the basal diet or the basal diet supplemented with organic and inorganic salts, chelating agents and diuretic compounds. The experimental criterion in these studies was rate of survival through 14 days.

Three studies were conducted to measure plasma NPN levels and kidney damage in rats receiving either the (a) basal choline-deficient diet (basal diet) [4]; (b) basal diet plus 0.7% NaCl (NaCl diet); (c) basal diet plus 2.23% $Na_2C_{10}H_{14}N_2O_8 \cdot 2H_2O$ (EDTA diet); or (d) basal diet plus 0.3% choline chloride (choline diet). NPN was determined by the method of Koch and McMeekin (5) and kidney damage by gross examination.

In a third experiment, groups of 15 rats were fed either (a) the basal diet; (b) the NaCl diet; or (c) a low mineral diet deficient in sodium and choline, which differed from the diet in table 1 in that it furnished only 26.5 g of $CaHPO_4$, no $NaHCO_3$, and 3.0 g of KCl/kg of diet (sodium-deficient diet). Folacin was also omitted from this diet because the folacin solution normally added to our diets contains $NaHCO_3$. The sodium-deficient diet contained less than 0.01% Na and there is a possibility that the diet may have been limiting in potassium. Based on the normal intake of the sodium-deficient diet, the rats received 13 to 14 mg of potassium/day from the salt mixture plus an undetermined amount from the other ingredients. The reported (6) daily potassium requirement for rats of the age used in this experiment is approximately 15 mg/day. This diet is adequate in calcium and phosphorus (6). Five rats fed the choline diet were used as a control group in this experiment.

The initial average weight of the rats fed the 4 diets was approximately 70 g. The rats were maintained on test for 14 days and weighed daily to determine the effect of diet on growth.

In a fourth phase of this work, five of the diets were fed to rats in special metabolism cages to facilitate the daily collection of urine and measurement of feed intake. The diets used were (a) basal diet; (b) NaCl diet; (c) EDTA diet; (d) sodium-deficient diet; and (e) choline diet. The diets were fed to 3 rats in individual cages for 7 days. The urine was collected in flasks containing toluene as a preservative. Urine was filtered, diluted, and analyzed for sodium, potassium, calcium, and iron. Sodium, potassium, and calcium were determined by flame photometry and iron by atomic absorption spectrophotometry.

RESULTS AND DISCUSSION

Some 840 weanling rats received the basal diet or the basal diet supplemented with different salts, organic compounds, chelating agents, and diuretics. Of this number, 358 had an initial weight below 75 g and 482 above 75 g. The survival rate was 48% for the lighter rats and 73% for the heavier rats. Thus body weight (or previous diet since the heavier rats generally had received the stock diet for a longer period of time) in itself affected survival. This is in agreement with

[4] In subsequent discussions, the diets will be identified by the term enclosed in parentheses.

the work of Griffith (7) in which he demonstrated that rats weighing over 69 g had a greatly increased survival rate when fed a choline-deficient diet. Several of the more significant results are presented in table 2. The weight of the rats in the heavy group ranged from 75 to 119 g and the lighter group from 45 to 74 g. The survival of the 2 groups fed the basal diet again demonstrates the effect of weight (or length of time fed previous diet) on survival. The addition of NaCl or EDTA to the basal diet increased survival. However, none of these diets prevented hemorrhagic kidney damage in the rats but simply appeared to decrease the mortality rate from this damage. Sodium chloride was more protective in the heavier rats. The protective action of NaCl does not appear to be a simple sodium effect since both the NaCl diet and the diet containing 1.66% $NaH_2PO_4 \cdot H_2O$ contained 0.466% of supplemental sodium.

TABLE 2

Effect of inorganic salts and EDTA on the survival of young rats fed a choline-deficient diet for 14 days

Diet	No. of rats/ treatment	Survival
		%
Initial weight below 75 g		
Basal [1]	56	46
Basal + 0.7% NaCl	16	69
Basal + 1.66% $NaH_2PO_4 \cdot H_2O$	11	55
Basal + 2.23% EDTA [2]	26	81
Initial weight above 75 g		
Basal [1]	52	62
Basal + 0.7% NaCl	17	88
Basal + 1.66% $NaH_2PO_4 \cdot H_2O$	14	57
Basal + 2.23% EDTA [2]	12	75

[1] See table 1.
[2] $Na_2EDTA \cdot 2H_2O$.

The data in table 3 demonstrate the effect of the basal, NaCl, EDTA, and choline diets on plasma NPN levels and gross kidney damage at 7 and 12 days. Rats fed the choline-deficient diet (basal diet) exhibited a tenfold increase in plasma NPN and severe kidney hemorrhage at 7 days. Similar NPN levels in choline-deficient rats have been reported previously from this laboratory (1). The addition of NaCl to the basal diet produced a small but consistent decrease in plasma NPN but it did not appreciably alter the degree of kidney damage at 7 days. Those rats that survived for 12 days with these 2 diets had NPN values comparable to normal levels. The rats receiving the EDTA had much lower NPN levels at 7 days and almost no kidney damage. However, at 12 days these rats had the highest NPN levels and extensive renal hemorrhage. It appears that EDTA did not prevent kidney damage but simply delayed it past 7 days; this delay may be of critical importance in the survival of young rats fed a choline-deficient diet.

One of the most striking discoveries in these studies was the observation that 70-g rats fed a low mineral, sodium-deficient, choline-deficient diet had over 90% survival rates through 14 days and had very mild kidney damage. It occurred to the investigators that growth rate during the early part of the experiment (first 5 or 6 days) might be involved in the increased survival of the rats fed the sodium-deficient diet. Thus an experiment was initiated to relate growth of rats receiving the

TABLE 3

Plasma NPN levels and degree of kidney damage

Diet	Exp. period	Plasma NPN level			Kidney hemorrhage [2]	
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2
	days	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*		
Basal [1]	7	270.0	265.4	284.2	2.0	1.8
NaCl	7	235.4	237.3	232.8	2.2	1.6
EDTA	7	81.3	83.4	163.9	0.6	0.6
Choline	7	20.4	26.4	22.9	0	0
Basal	12	—	47.4	—	—	1.6
NaCl	12	—	41.9	—	—	1.2
EDTA	12	210.3	67.2	—	2.0	2.2
Choline	12	23.2	23.8	—	0	0

[1] See table 1.
[2] Given on a scale in which zero is no damage and 3 is severe damage.

TABLE 4

Effect of diet on daily gain during the first 5 days of the experiment and the relationship of gain to survival in choline-deficient rats

Diet	No. of rats/ treatment	Avg initial wt	14-day survival	Avg daily gain [1]
		g	%	g
Basal	15	70.3	47	5.1 ± 0.9 [2]
Sodium-deficient	15	70.2	93	2.8 ± 0.8
NaCl	15	70.3	53	5.1 ± 0.6
Choline	5	70.2	100	5.8 ± 1.0

[1] The average gain is for the first 5 days of the experiment.
[2] Mean ± SD.

different diets to their 14-day survival rate. This study is summarized in table 4. Daily gains given in the table are for the first 5 experimental days. There were no significant differences in the 5-day daily gains among groups of rats fed the basal, NaCl, and choline diets; however, the sodium-deficient diet caused a marked decrease in daily gain. Only one rat died in the sodium-deficient group and that was a rat that gained 5.2 g/day — a rate similar to those receiving the basal and NaCl diets. Renal damage due to choline deficiency apparently is related to rate of growth during the first 5 days of the experiment and diets which suppress growth during this period are renoprotective as evidenced by the 14-day survival of the sodium-deficient rats. As noted in the experimental procedure, the sodium-deficient diet also contained less calcium, phosphorus, and potassium than the basal diet. Two other sodium-deficient diets were tested to determine whether the decreased growth rate was the result of either (a) the sodium deficiency alone, (b) the combined effect of the decreased Na, K, Ca, and P content of the diet, or (c) the decreased K content. Rats weighing 60 g were used in this experiment and the daily gains of rats fed the basal and sodium-deficient diets were again determined. One of the diets was identical to the basal diet except that $NaHCO_3$ was replaced by sucrose. Daily gain for the first 5 days with this diet was 2.8 ± 0.5 g.[5] The second diet was equivalent to the original sodium-deficient diet except that the KCl was increased to 6.0 g/kg. The average 5-day daily gain was 2.3 ± 0.4 g. The average daily gain for rats fed the basal diet was 4.3 ± 0.4 g and for the sodium-deficient diet 2.3 ± 0.3 g. The decreased gain apparently was related to the sodium content of these diets. The performance of rats fed the first diet also indicates that omission of folacin from the sodium-deficient diet was not a factor in the decreased growth rate.

The average daily feed intakes of rats fed the basal, NaCl, EDTA, sodium-deficient and choline diets are shown in figure 1. The rats fed the choline diet had the highest feed intake, whereas those fed the EDTA and the sodium-deficient diets had the lowest intake. There was a substantial decrease in the intake of all diets after 5 days except for the rats fed the choline

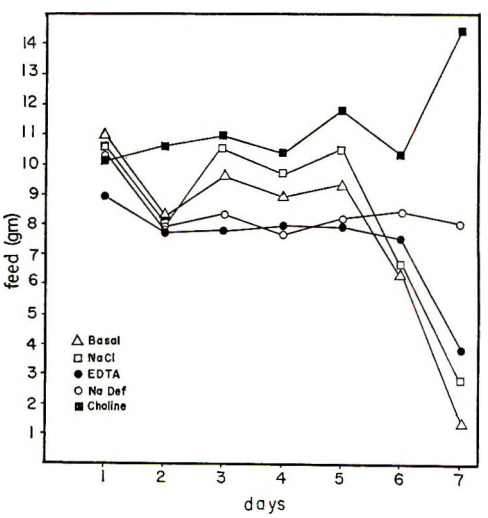

Fig. 1 Average daily feed intake for male weanling rats fed the following diets: basal = choline-deficient (see table 1); NaCl = basal plus 0.7% NaCl; EDTA = basal plus 2.23% EDTA; Na def. = sodium-deficient, choline-deficient; choline = basal plus 0.3% choline Cl.

[5] Mean ± SD.

and sodium-deficient diets. The similarity in the feed intakes of rats fed the EDTA and sodium-deficient diets prompted an investigation of the growth rate of rats fed the EDTA diet during the first 5 experimental days. The rats receiving the EDTA diet gained 3.0 ± 0.6 g during this period (compared with 2.3 ± 0.3 g for the sodium-deficient diet). These results suggest that the protection afforded by the sodium-deficient diet is not a specific sodium effect; rather, any dietary addition or deletion that lowers intake of the choline-deficient diet and thus decreases daily gain will likely increase 14-day survival. Figure 2 illustrates the average daily urine volume for the rats fed the 5 diets. The NaCl diet was definitely diuretic as was the sodium-deficient diet, although to a lesser degree. The EDTA diet suppressed urine output; however, the animals fed this diet had mild diarrhea. The urine volume of rats that received the basal, NaCl, and EDTA diets tended to decrease as the experiment progressed, whereas that of rats fed the sodium-deficient and choline diets increased. The daily sodium and calcium excretion of the rats receiving the EDTA was equal to that of the rats fed the basal diet, although the EDTA rats had a markedly lower urine volume. The daily urinary excretion of iron was low and variable (10–70 μg); however, it appeared that rats receiving the EDTA had the highest daily excretion of iron of all the groups.

Figure 3 presents the daily urinary excretion of sodium. The NaCl diet caused an elevated sodium excretion through 5 days. The decrease after 5 days corresponds to a decrease in feed intake (fig. 1). The rats fed the sodium-deficient diet had no detectable sodium in their urine.

A relationship was observed between urinary sodium and calcium excretion. The curves for sodium excretion by rats fed the NaCl diet (fig. 3) and for calcium excretion (fig. 4) are almost identical. Furthermore, despite the adequate calcium in the sodium-deficient diet, rats fed this diet had no measurable excretion of calcium. A similar relationship between the renal excretion of calcium and sodium has been observed previously in dogs by Walser (8) and Wesson (9).

Figure 5 shows the daily urinary excretion of potassium. Rats fed the NaCl diet had the highest potassium excretion through the first 5 days, whereas animals receiving the EDTA and sodium-deficient diets had the lowest. Rats fed the choline and sodium-deficient diets showed a definite increase after the fifth day.

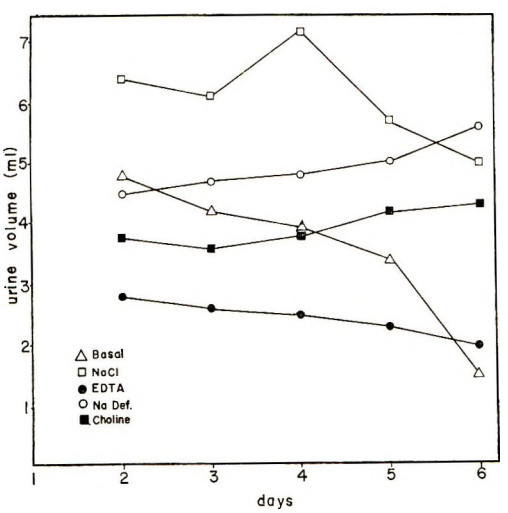

Fig. 2 Average daily urine volume for male weanling rats fed the following diets: basal = choline-deficient (see table 1); NaCl = basal plus 0.7% NaCl; EDTA = basal plus 2.23% EDTA; Na def. = sodium-deficient, choline-deficient; choline = basal plus 0.3% choline Cl.

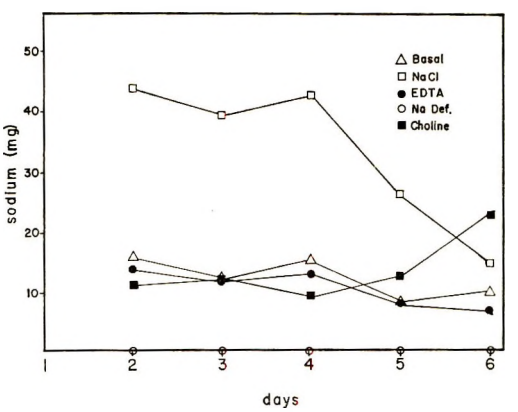

Fig. 3 Average daily urinary excretion of sodium for male weanling rats fed the following diets: basal = choline-deficient (see table 1); NaCl = basal plus 0.7% NaCl; EDTA = basal plus 2.23% EDTA; Na def. = sodium-deficient, choline-deficient; choline = basal plus 0.3% choline Cl.

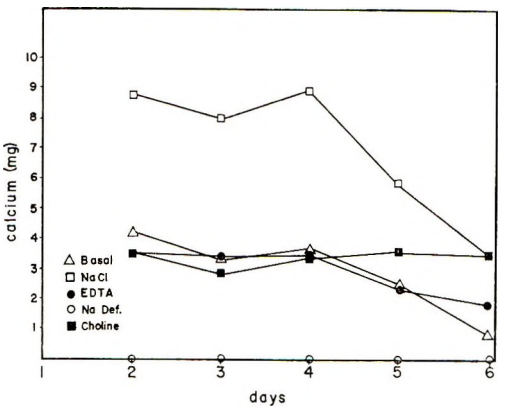

Fig. 4 Average daily urinary excretion of calcium for male weanling rats fed the following diets: basal = choline-deficient (see table 1); NaCl = basal plus 0.7% NaCl; EDTA = basal plus 2.23% EDTA; Na def. = sodium-deficient, choline-deficient; choline = basal plus 0.3% choline Cl.

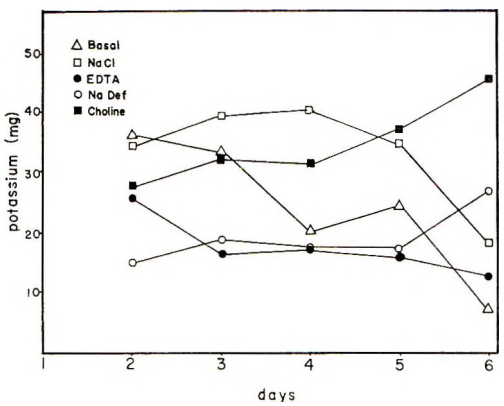

Fig. 5 Average daily urinary excretion of potassium for male weanling rats fed the following diets: basal = choline-deficient (see table 1); NaCl = basal plus 0.7% NaCl; EDTA = basal plus 2.23% EDTA; Na def. = sodium-deficient, choline-deficient; choline = basal plus 0.3% choline Cl.

CONCLUSIONS

The effect of alterations in the mineral composition of a choline-deficient diet on the survival of weanling rats during the first 14 days of receiving the diet is exceedingly variable. It is apparently related to such factors as weight of the rats, growth rate during the early days of the experiment, time at which the renal hemorrhage occurs (by the seventh day or after) and possibly the diuretic action of the diet. In several of the experiments, it appears that the effect of addition of a chelating agent or deletion of a mineral on survival is not related so much to its effect on mineral metabolism and renal function as it is to its effect on feed intake and growth. However, the protection furnished by NaCl cannot be explained on this basis, but it may be related to its diuretic action or its effect on the renal excretion of sodium, calcium, and potassium. The small but consistent decrease in plasma NPN levels exhibited by rats fed the NaCl diet (table 3) may be related to increased urine flow. It should be noted that the protection furnished by NaCl was inconsistent and highly variable. Weanling rats receiving the choline-deficient diet used in these experiments entered a physiological crisis on about the seventh experimental day. This crisis involves the hemorrhagic degeneration of the kidneys, which is fatal in approximately 60% of the rats. Apparently the dietary alterations reported in these studies did not prevent hemorrhagic renal damage but rather, mediated the crisis or delayed it to a time when the animal was better able to cope physiologically with renal dysfunction. Apparently there is a very limited period (up to approximately one month of age) in which a significant number of male rats succumb to the acute effects of hemorrhagic kidney damage produced by choline deficiency. Rats that survive this period or are fed the diet at an older age may live for many months when fed the choline-deficient diet.

ACKNOWLEDGMENTS

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Estimation of the Extent of Conversion of Dietary Zein to Microbial Protein in the Rumen of Lambs [1]

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ABSTRACT A metabolism experiment was conducted to estimate the extent of conversion of dietary nitrogen to microbial protein in the rumen of lambs fed purified diets. Zein was fed as the nitrogen source with 2 cellulose-to-starch ratios. Physical fractionation procedures showed 26.3 and 30.5% microbial protein in the abomasal fluid of lambs fed the high cellulose and high starch diets, respectively. Based on the changes of lysine ratios between the diet and abomasal fluid, the percentage microbial protein was estimated to be 28.0 and 23.8 with the high cellulose and high starch diets, respectively. Using the estimates of conversion based on physical separation of abomasal fluid protein, and using the amino acid compositions of zein and microbial protein, a predicted amino acid composition of abomasal fluid with the 2 diets was calculated. Predicted values were similar to determined values, indicating that amino acid analyses of abomasal fluid might be used to estimate conversion when homologous proteins are fed to ruminants.

The fermentation process in the rumen allows ruminants to utilize poor quality proteins and nonprotein nitrogen compounds. If the conversion of dietary nitrogen to microbial protein were complete, the nature of the dietary nitrogen would be of little significance in ruminant nutrition. However, the reports of McDonald (1) and McDonald and Hall (2) indicate that the extent of conversion can vary considerably among different dietary nitrogen sources. Johnson et al. (3) suggest that nitrogen feeds which are converted to microbial protein in the rumen have similar biological values, whereas those nitrogen feeds not utilized by rumen microorganisms differ in biological value as when fed to nonruminants.

Dietary carbohydrates can play an important role in conserving the ammonia released in the rumen from dietary nitrogen (4, 5). If a dietary carbohydrate is degraded by rumen microorganisms at a rate comparable to the ammonia release from a poor quality dietary nitrogen source, maximal microbial protein synthesis should occur, resulting in more efficient nitrogen utilization by the animal.

The present research was initiated to study the effect of 2 cellulose-to-starch ratios on the extent of conversion of dietary zein to microbial protein with the changes in amino acid ratios between diet and abomasal fluid being observed.

EXPERIMENTAL PROCEDURES

Six crossbred wether lambs, weighing 35 kg and fitted with permanent abomasal cannulas (6), were fed the two purified diets described in table 1. Each diet was fed at the rate of 200 g/lamb twice daily. Thirty minutes were allowed for each lamb to consume the feed offered.

Following a 3-week dietary adjustment period, lambs were kept in individual stanchion-type metabolism crates. The adjustment period was followed by a 6-day experimental period in which samples of abomasal fluid, feces and urine were taken. Samples of abomasal fluid were collected at each 2-hour interval after both the AM and PM feedings and feces and urine were collected every 24 hours. Treatments were then reversed and the procedures repeated. This arrangement provided 6 observations per treatment.

The extent of conversion of dietary zein to microbial protein was estimated by methods similar to those described by

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[2] Present address: Fort Hays Agricultural Experiment Station, Hays, Kansas.

TABLE 1

Composition of diets fed to lambs

	Diet	
	High cellulose	High starch
	%	%
Zein [1]	12.8	12.8
Glucose	10.0	10.0
Cellulose [2]	49.8	28.5
Cornstarch	21.4	42.7
Minerals [3]	6.0	6.0

[1] Purified preparation, 91% crude protein.
[2] Solka Floc BW-40 (Brown Company, Berlin, New Hampshire), a purified wood cellulose.
[3] Composed of: (in per cent) NaCl, 0.87; K_2HPO_4, 1.12; KCl, 0.73; $CaHPO_4 \cdot 2H_2O$, 1.30; $MgSO_4$, 0.3495; $CaSO_4 \cdot 2H_2O$, 1.478; $CaCO_3$, 0.107; $FeSO_4$, 0.0275; KI, 0.0028; $ZnSO_4$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.0011; $CoSO_4 \cdot H_2O$, 0.0006; CaF_2, 0.0008; and $MnSO_4$, 0.0050.

McDonald (1). A diagram of the fractionation of nitrogen components in abomasal fluid is shown in figure 1. Based on the assumptions of McDonald (1) concerning loss of nitrogen from the rumen, forms of nitrogen leaving the rumen and pepsin activity in the abomasum, the percentages of the different nitrogen and protein fractions of abomasal fluid were calculated from the following equations:

$$\% \text{ Zein N} = \frac{\text{ethanol-soluble N} - \text{TCA-soluble N}}{\text{total N}} \times 100$$

$$\% \text{ Non-zein N} = \frac{\text{total N} - \text{zein N}}{\text{total N}} \times 100$$

$$\% \text{ NPN} = \frac{\text{TCA-soluble N}}{\text{total N}} \times 100$$

$$\text{Total protein} = (\text{total N} - \text{NPN}) \times 6.25$$

$$\% \text{ Zein protein} = \frac{\text{zein N} \times 6.25}{\text{total protein}} \times 100$$

$$\% \text{ Non-zein protein} = \frac{\text{total protein} - \text{zein protein}}{\text{total protein}} \times 100.$$

Amino acids in hydrolysates of diet and abomasal fluid were determined by automated ion-exchange chromatography procedures (8). Samples were subjected to hydrolysis in 6 N HCl by methods described by Block and Weiss (7).

Statistical analyses were conducted according to the procedures set forth by Steel and Torrie (9).

RESULTS

The nitrogen digestibility and retention results are presented in table 2. No dietary differences were noted for apparent nitrogen digestibility. The low apparent digestibility indicates that the dietary zein nitrogen was not efficiently utilized. All lambs were in negative nitrogen balance, indicating that the zein nitrogen was not utilized to a large enough extent to maintain nitrogen equilibrium.

The percentage zein and non-zein proteins in abomasal fluid of lambs at different times after feeding the 2 diets are shown in table 3. No significant dietary differences were noted. The mean percentage non-zein protein in abomasal fluid was 26.3 and 30.5 with the high cellulose and high starch rations, respectively. These results indicate that microbial protein synthesis was occurring in the rumen; however, the small extent of conversion supports the observation of low digestibility and negative nitrogen balance with both diets (table 2).

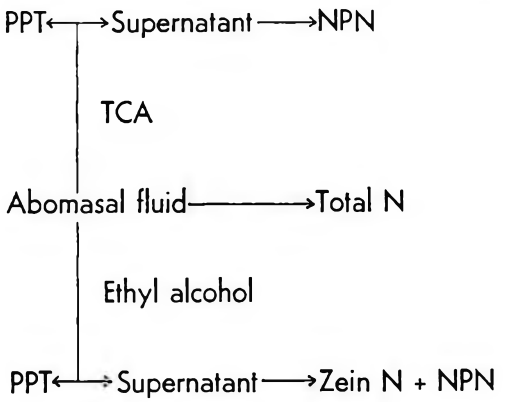

Fig. 1 Fractionation scheme for determining zein and non-protein nitrogen in abomasal fluid.

TABLE 2

Nitrogen digestibility and retention of lambs fed two cellulose-to-starch ratios with zein [1]

	Diet	
	High cellulose	High starch
N intake/day/lamb, g	7.5	6.7
Fecal N, g	4.5	4.1
Urinary N, g	3.3	2.7
Apparent digestibility, %	40.0	38.8
N retained, g	−0.3	−0.1
N retained, % of intake	−4.0	−1.5
N retained, % of digested	−10.0	−3.8

[1] Six animals/group.

TABLE 3

Zein and non-zein protein in abomasal fluid of lambs at different times after feeding two cellulose-to-starch ratios with zein

	Hours after feeding						
	0	2	4	6	8	10	Mean
High cellulose diet:							
Zein protein, %	76	71	75	72	81	67	73.7
Non-zein protein, %	24	29	25	28	19	33	26.3
High starch diet:							
Zein protein, %	64	78	75	73	63	64	69.5
Non-zein protein, %	36	22	25	27	37	36	30.5

A comparison of the essential amino acids, except tryptophan, in the diet and abomasal fluid of lambs fed the 2 cellulose-to-starch ratios is presented in table 4. The dietary amino acids, except methionine, were significantly different in concentration from the amino acids in abomasal fluid of lambs fed the high cellulose ration. With the high-starch diet, methionine and isoleucine were the only amino acids not significantly different between diet and abomasal fluid. Leucine, methionine and phenylalanine were less concentrated in abomasal fluid than in the diet. Molar percentage lysine differed to the greatest extent between diet and abomasal fluid.

Table 5 shows the molar percentage of the essential amino acids in bacterial protein and that observed in abomasal fluid of lambs fed the 2 diets in this study. Concentrations of all the abomasal fluid amino acids, except histidine, were significantly different from that of bacterial protein. The results shown in tables 4 and 5 suggest that the amino acids present in abomasal fluid with both feeding regimens resulted from a mixture of dietary and microbial proteins being present in the fluid.

If the amino acids in abomasal fluid are assumed to be primarily of dietary or microbial origin (1), the validity of conversion estimates based on physical separation could be checked by comparing the actual amino acid composition with a predicted amino acid pattern calculated for mixture of dietary and microbial proteins. Using the estimates of conversion based

TABLE 4

Essential amino acids recovered in the ration and abomasal fluid of lambs fed two cellulose-to-starch ratios with zein

	Essential amino acids recovered		
	Diet	Abomasal fluid	
		High cellulose diet	High starch diet
	molar %	*molar %*	*molar %*
Threonine [b,c]	2.6	3.7	3.7
Valine [b,c]	4.2	5.4	5.2
Methionine [a]	1.3	1.0	1.2
Isoleucine [b]	4.1	4.8	4.5
Leucine [a,b,c]	20.6	16.7	18.1
Phenylalanine [a,b,c]	5.3	4.7	5.0
Lysine [a,b,c]	0.1	1.8	1.5
Histidine [b,c]	0.9	1.1	1.2
Arginine [b,c]	1.0	1.7	1.7

[a] Significant difference ($P < 0.05$) between high cellulose and high starch abomasal fluid amino acids.
[b] Significant difference ($P < 0.05$) between diet and high cellulose abomasal fluid amino acids.
[c] Significant difference ($P < 0.05$) between diet and high starch abomasal fluid amino acids.

TABLE 5

Essential amino acids recovered in bacterial protein and abomasal fluid of lambs fed two cellulose-to-starch ratios with zein

	Essential amino acids recovered		
	Bacterial protein [1]	Abomasal fluid	
		High cellulose diet	High starch diet
	molar %	*molar %*	*molar %*
Threonine [a,b]	7.0	3.7	3.7
Valine [a,b]	6.7	5.4	5.2
Methionine [a,b]	1.5	1.0	1.2
Isoleucine [a,b]	6.4	4.8	4.5
Leucine [a,b]	7.0	16.7	18.1
Phenylalanine [a,b]	3.8	4.7	5.0
Lysine [a,b]	6.5	1.8	1.5
Histidine	1.2	1.1	1.2
Arginine [a,b]	3.2	1.7	1.7

[a] Significant difference ($P < 0.05$) between bacterial protein and high cellulose abomasal fluid amino acids.
[b] Significant difference ($P < 0.05$) between bacterial protein and high starch abomasal fluid amino acids.
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TABLE 6

Comparison of actual and predicted amino acids in abomasal fluid of lambs fed two cellulose-to-starch ratios with zein

	Amino acids in abomasal fluid			
	High cellulose diet		High starch diet	
	Actual	Predicted [1]	Actual	Predicted [1]
	molar %	*molar %*	*molar %*	*molar %*
Threonine	3.7	3.8	3.7	4.0
Valine	5.4	5.0	5.1	4.9
Isoleucine	4.8	4.7	4.5	4.8
Leucine	16.7	17.0	18.1	16.4
Methionine	1.0	1.3	1.1	1.3
Phenylalanine	4.7	4.9	5.0	4.9
Lysine	1.8	1.8	1.5	2.1
Histidine	1.1	1.0	1.2	1.0
Arginine	1.7	1.5	1.7	1.7

[1] (100) (% amino acid in abomasal fluid) = (% zein) (% amino acid in zein) + (% bacterial protein) (% amino acid in bacterial protein).

on physical separation (table 3) and the amino acid compositions of zein and microbial protein, the predicted amino acid composition of abomasal fluid with the 2 diets was calculated. These values are shown in table 6. The similarity between the actual and predicted values verifies the validity of physical separation procedures and suggests that information relative to the amino acid ratios in the diet, microbial protein and abomasal fluid might be used to estimate conversion of dietary protein to microbial protein. Since the differences between diets and abomasal fluids were greatest for lysine, it was selected as an indicator of the extent of conversion. Values for non-zein proteins in abomasal fluid calculated from lysine ratios at different times after feeding are shown in table 7. The similarity of results with the 2 methods of estimating conversion (tables 3 and 7) suggests the possibility of estimating the extent of conversion of homologous dietary proteins from amino acid analyses of abomasal fluid.

TABLE 7

Non-zein protein in abomasal fluid of lambs calculated from lysine ratios

Hours after feeding	Calculated non-zein protein in abomasal fluid	
	High cellulose diet	High starch diet
	%	%
0	23.9	23.8
2	31.7	20.2
4	27.9	27.2
6	26.3	25.6
8	26.7	25.3
10	31.3	20.6
Mean [1]	28.0	23.8

[1] Significant difference ($P < 0.05$) between diets.

DISCUSSION

For a poor-quality protein to be utilized efficiently by ruminants, it must be hydrolyzed in the rumen and the nitrogen used by the microbial population for synthesis of higher quality microbial protein (4). Although indicating that zein was not extensively converted, conversion estimates from this study show that significant quantities were converted to microbial protein. The changes that were noted between the amino acid compositions of the diet and abomasal fluid provide further evidence of the important role of the microbial population in affecting the protein nutrition of ruminants.

The "predicted" amino acid values with different ratios of zein and microbial protein suggest the type of problem that may be encountered in attempting to meet the essential amino acid requirement of the tissue if a poor quality protein, such as zein, is not extensively converted to microbial protein. With poor conversion, characteristic amino acid patterns of such dietary proteins could be expected to affect the nutritional status of the animal; however, with complete conversion, the amino acid make-up of the dietary protein would be of little significance. The ex-

tent of conversion would be less critical with a protein of quality similar to that of microbial protein because a balanced mixture of amino acids would be subject to absorption whether the protein was converted in the rumen or passed out of the rumen unattacked by the rumen microorganisms.

The carbohydrate ratios in this study appeared to have little influence on the conversion of zein to microbial protein. The work of McDonald (10) suggests that zein is poorly converted because it is resistant to proteolysis by rumen microorganisms, resulting in a slow release of ammonia (10). Likewise, the major influence of dietary carbohydrates on rumen metabolism of nitrogen has been reported to be in utilizing the ammonia released from a readily soluble nitrogen source to synthesize cell protein and not in the rate of proteolysis of feed nitrogen sources (11). It may be surmised that the rate of proteolysis rather than uptake of released nitrogen limits conversion of zein to microbial protein.

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Effect of Vitamin D_3 on the in vitro Transport of Calcium by the Chick Intestine [1]

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ABSTRACT The effect of vitamin D_3 treatment of the chick on concentrative transport of calcium by intestinal loops in vitro, on intestinal permeability to calcium in vitro and on calcium efflux from tied-off intestinal loops in situ was determined. Concentrative transport of calcium in vitro could not be demonstrated when chick intestine was incubated in K-H buffer (Na = 145 mM) but was observed when everted intestinal loops were incubated in a low sodium buffer (Na = 26 mM). In this low sodium medium, concentrative transport of calcium was observed only in the ileal segment and was greatly enhanced by vitamin D_3 treatment of the chick. Vitamin D_3 treatment was also shown to increase the permeability to calcium of the intestinal loop in vitro. In the in vitro preparations calcium was not transferred across the wall of the duodenum. In situ, however, the most rapid efflux of calcium occurred in the duodenal loop and was greatly enhanced by vitamin D treatment.

The concentrative transport of calcium by the everted intestine in vitro has been studied extensively in the rat, rabbit, guinea pig, mouse and hamster (1–3). In these animals, except for the hamster, the duodenum was found capable of transporting calcium against a concentration gradient when buffers containing approximately 150 mM sodium were used. Under such conditions, the serosa is electrically positive with respect to the mucosa (4) and hence calcium is transported against an electrochemical potential gradient. This concentrative transport of calcium was shown both by Schachter et al. (1, 5) and by Harrison and Harrison (2) to be enhanced by vitamin D treatment of the animal from which the intestine was taken. Such observations led Schachter to suggest that the physiological role of vitamin D is to promote the active transport of calcium in the intestine. Harrison and Harrison (6), however, using an intestinal preparation in which active ion transport was inhibited by N-ethylmaleimide, showed that vitamin D treatment increased the permeability of the intestine to calcium.

Despite the usefulness of the chick as an experimental animal for calcium metabolism studies, there is little information on calcium transport by chick intestine in vitro. Sallis and Holdsworth (7) and Holdsworth (8) measured the uptake and release of ^{45}Ca from the intestine in vitro in an effort to explain the mode of action of vitamin D. There are no reports of mucosal to serosal transport of calcium by the chick intestine in vitro.

METHODS

One-day-old white leghorn male chicks were obtained from a commercial hatchery, placed immediately in an electrically heated battery brooder, and fed the vitamin D-deficient diet shown in table 1. This diet was designed to supply all nutrients including calcium and phosphorus at conventional levels but with no supplementary vitamin D. The chicks were divided into 2 lots: one lot was fed up to 4 weeks the diet without further supplementation (rachitic) and the other received, by crop intubation, 1000 IU of crystalline vitamin D_3 in propylene glycol at 10-day intervals (vitamin D-treated).

In vitro experiments. The chicks used for these experiments were between three and four weeks old. They were bled by heart puncture and anesthetized by the same route with sodium pentobarbital. The small intestine was removed, washed with

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TABLE 1

Composition of the experimental vitamin D-depletion chick diet

	%
Soybean oil meal (50% protein)	45.00
Glucose	47.00
Peanut oil, refined	3.00
Choline chloride	0.10
DL-Methionine	0.15
Vitamin mixture [1]	0.25
Salt mixture [2]	0.50
Potassium phosphate, monobasic	1.00
Sodium phosphate, monobasic	1.00
Calcium carbonate, precipitated	2.00

[1] Supplied/kg of ration: (in milligrams) thiamine, 25; riboflavin, 16; Ca pantothenate, 20; pyridoxine, 6; biotin, 0.6; folic acid, 4; menadione, 2; niacin, 150; ascorbic acid, 250; inositol, 100; vitamin B_{12}, 0.02; and vitamin A, 10,000 IU; and α-tocopherol, 5 IU.

[2] Supplied in mg/kg ration: NaCl, 2,877; $FeSO_4$, 286; $MgSO_4$, 1,000; $MgCO_3$, 500; $MnSO_4 \cdot H_2O$, 150; KI, 6; $CuSO_4$, 8; $ZnCO_3$, 150; and $Na_2MoO_4 \cdot 2H_2O$, 0.6.

physiological saline and placed in ice-cold 20 mM KCl-135 mM NaCl solution. It was then divided into the appropriate 11-cm segments, everted and incubated under 95% O_2, 5% CO_2 atmosphere as described by Harrison and Harrison (2).

The small intestinal loops were used in either of 2 systems: (a) the "concentrative transport system" in which initially the solutions bathing both the mucosal and serosal surfaces contained the same concentration of calcium and ^{45}Ca, 0.25 mM and 25 μCi/liter, respectively. In this system calcium transport was measured both as micromoles calcium transported into the serosal compartment per loop and by the ratio of the concentration of calcium in serosal fluid to that in mucosal fluid (C_s/C_m); and (b) the "permeability system" in which concentrative transport was inhibited by 0.6 mM N-ethylmaleimide in the buffer, 2.5 mM calcium and 25 μCi of ^{45}Ca per liter were added to the mucosal solution only and the quantity of ^{45}Ca entering the initially calcium free serosal phase was taken as the index of calcium permeability. The results are expressed as micromoles calcium transferred per loop per hour. The incubation times were 90 and 60 minutes, for the transport and permeability experiments, respectively.

Harrison and Harrison (9) have shown that concentrative transport of calcium in vitro by rat ileum occurred only if the everted ileal loops were incubated in a low sodium medium. A low sodium medium (Na = 26 mM) was also tried in the present study with chick intestine to see if the same effect could be demonstrated in the chick and to find a suitable in vitro system for assaying vitamin D activity in the chick intestine. The incubation medium in this experiment contained in millimoles per liter: mannitol, 198; KCl, 25; NaH_2PO_4, 1.2; $NaHCO_3$, 25; $MgSO_4$, 1.2; glucose, 20; and $CaCl_2$, 0.25; with 25 μCi of ^{45}Ca per liter.

In situ experiment. The unidirectional efflux of ^{45}Ca from various intestinal segments in situ was determined as an estimate of the rate of absorption of calcium by the respective segments in vivo. Three-week-old rachitic and vitamin D-treated chicks were anesthetized by intravenous sodium pentobarbital and the left abdomen was cleared of feathers. A longitudinal incision was made just above the sternum, through the skin and peritoneum. The duodenum, lower jejunum and lower ileum were identified and 8-cm portions were separated from these segments leaving the blood vessels intact. These portions were flushed with physiological saline and air. One side was then tied off and a loose ligature placed on the other end. The buffer was introduced by use of a syringe with a blunt needle inserted through this end and the ligature was tightened around the needle. Four-tenths milliliter of the ^{45}Ca-containing buffer were injected and the ligature was tightened completely as the needle was withdrawn. After injecting the three intestinal portions, the skin and peritoneum were sutured and incubation was allowed for 20 minutes. The buffer contained in millimoles per liter: NaCl, 126; KCl, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 10; $CaCl_2$, 6.5; glucose, 20; and ^{45}Ca, 250 μCi per liter.

At the end of the incubation, each portion of intestine was removed and placed in a crucible. The samples were then dried, ashed, dissolved in N HCl and ^{45}Ca was determined in each sample. The unidirectional efflux is defined as the amount of ^{45}Ca introduced minus the residual. In trial experiments it was found that the influx of ^{45}Ca under these conditions is insignificant and can therefore be neglected.

RESULTS

The results of the transport experiments are shown in table 2. When everted intestinal segments were incubated in the standard Krebs-Henseleit buffer, calcium was lost from the serosal solutions in all segments tested, in both rachitic and vitamin D-treated chicks. These losses resulted in C_s/C_m ratios lower than unity. The C_s/C_m ratio decreased along the intestine from proximal to distal direction in both rachitic and vitamin D-treated chicks. There was, however, a small but consistent difference between the 2 groups with the C_s/C_m ratio being higher in the vitamin D-treated animals. There was little change in serosal volume in the duodenum and upper jejunum, but there was a marked mucosal to serosal fluid transport in the lower jejunum and ileum.

Because of lack of concentrative transport of calcium by chick intestine in vitro in the medium containing 145 mM sodium, studies with the low sodium buffer were made. The results are included in table 2. Water transport into the serosal phase was markedly reduced by reduction of the sodium concentration of the medium. As compared with the results with Krebs-Henseleit buffer the net loss of calcium from the serosal solution was reduced in the segments from rachitic chicks resulting in higher C_s/C_m ratios. In the vitamin D-treated animals, there was a significant mucosal to serosal transport of calcium in the ileal loop and a C_s/C_m ratio which approached two. Incubation in a low sodium buffer, therefore, resulted in concentrative calcium transport by the everted ileum of vitamin D-treated chicks.

Results of the permeability experiments are shown in table 3. N-Ethylmaleimide in a 0.6 mM concentration eliminated water transport in the ileum, but there was still some water transport in the jejunum. Under these in vitro conditions, the wall of the chick duodenum was found to be almost totally impermeable to calcium. The intestinal permeability to calcium was highest in the jejunum and somewhat lower in the ileum. In both jejunum and ileum, vitamin D treatment significantly increased the calcium permeability.

The in situ experiment (table 4) indicated that in the rachitic chick there was no significant difference among the various intestinal segments with respect to calcium efflux. Vitamin D treatment considerably increased the efflux from all segments, but most markedly from the duodenum in which it was about tripled.

DISCUSSION

The in situ experiment (table 4) indicated that the in vivo absorption of calcium in the vitamin D-treated chick is highest in the duodenum, in agreement with similar observations in the rat (10).

TABLE 2

Calcium transport across chick-intestinal wall in vitro [1]

Segment	Rachitic			Vitamin D-treated		
	ΔV [2]	ΔCa [3]	C_s/C_m [4]	ΔV	ΔCa	C_s/C_m
	ml	*μM/90 min*		*ml*	*μM/90 min*	
			Krebs-Henseleit buffer			
Duodenum	−0.01	−0.066	0.66 ± 0.13 [5]	0.11	−0.043	0.74 ± 0.16
Jejunum, upper	0.11	−0.055	0.64 ± 0.18	0.02	−0.065	0.73 ± 0.24
Jejunum, lower	0.22	−0.042	0.44 ± 0.06	0.20	−0.022	0.51 ± 0.06
Ileum, upper	0.51	−0.041	0.37 ± 0.09	0.31	−0.049	0.50 ± 0.12
Ileum, lower	0.38	−0.071	0.28 ± 0.05	0.30	−0.047	0.45 ± 0.08
			Low-sodium buffer			
Duodenum	0.00	−0.058	0.64 ± 0.10	−0.05	−0.071	0.83 ± 0.18
Jejunum, lower	−0.05	−0.055	0.71 ± 0.07	−0.08	−0.007	0.90 ± 0.29
Ileum, lower	0.16	−0.020	0.67 ± 0.24	0.01	0.061	1.97 ± 0.78

[1] Everted loops of designated segments of small intestine incubated for 90 minutes at 37°.
[2] Change in volume of serosal fluid during incubation, initial volume 0.6 ml.
[3] Quantity of calcium added to or removed from serosal fluid during incubation.
[4] The ratio of the concentration of calcium in serosal fluid to that in mucosal fluid at end of incubation (initial $C_s/C_m = 1$).
[5] The data are averages of at least 5 experiments ± SD.

TABLE 3

Effect of vitamin D_3 on calcium permeability of chick intestine in vitro [1]

	Rachitic		Vitamin D-treated	
	ΔV [2]	ΔCa [3]	ΔV	ΔCa
	ml	*μM*	*ml*	*μM*
Duodenum	−0.02	0.035 ± 0.035 [4]	−0.04	0.035 ± 0.038
Jejunum, lower	0.18	0.247 ± 0.091	0.27	0.417 ± 0.183
Ileum, lower	−0.03	0.189 ± 0.045	0.00	0.320 ± 0.036

[1] Permeability indicates micromoles calcium transported from the mucosal to serosal media by 10-cm everted gut loops. The loops were incubated for 60 minutes in Krebs-Henseleit bicarbonate buffer containing 0.6 mM N-ethylmaleimide. The mucosal solution contained 2.5 mM calcium and 25 μCi of ^{45}Ca/liter; the serosal solution was calcium-free at the start of the incubation.
[2] ΔV = change in serosal volume during incubation.
[3] ΔCa = quantity of calcium entering the serosal medium during incubation.
[4] The data are averages of 5 birds ± SD.

TABLE 4

Effect of vitamin D on outflow of ^{45}Ca from various segments of chick intestine in situ

Segment	^{45}Ca outflow	
	Vitamin D-treated	Rachitic
	%	%
Duodenum	48.0 [1] (33.5–57.0)	11.9 (9.5–14.8)
Jejunum	22.2 (19.6–24.4)	11.1 (5.3–14.6)
Ileum	16.9 (15.2–19.7)	9.5 (5.7–13.2)

[1] Percentage of the injected dose, with the range indicated in parentheses; 0.4 ml of buffer containing 5 mM calcium and 250 μCi of ^{45}Ca per liter were introduced into intestinal sacs with the blood supply intact and incubated to 20 minutes.

It was, therefore, unexpected to find that under the in vitro conditions the duodenum was impermeable to calcium (table 3), in contrast with observations in the rat (6). This lack of permeability may be due to thickness of the muscular coats of the tissue or to severe vasoconstriction under these conditions. Such a discrepancy between the calcium transport in vivo and in vitro calls for caution in the interpretation of in vitro determinations of transport rates.

The lack of concentrative transport of calcium by everted chick intestine in vitro when incubated in Krebs-Henseleit buffer stimulated the experiments in which the everted loops were incubated in a low sodium buffer. At a 26 mM sodium concentration, concentrative transport of calcium could be demonstrated with ileal loops although C_s/C_m ratios of greater than one were not developed by duodenal or jejunal loops. In the low sodium medium it was possible to demonstrate that vitamin D treatment of the animal greatly increased concentrative transport of calcium by everted intestinal loops. The chick ileum can therefore be used to demonstrate the effect of vitamin D on calcium transport by chick intestine.

The mechanism for the inhibitory effect of sodium on concentrative calcium transport by small intestine in vitro requires further investigation. Although the potential difference between serosal and mucosal compartments is altered by changes in sodium concentration of the bathing medium, preliminary observations indicate that the concentration difference of calcium between serosal and mucosal fluids is not directly related to the potential difference. In other systems a competition between sodium and calcium for transport across a membrane has been demonstrated. Niedergerke et al. (11) found a reciprocal relation between sodium concentration and uptake of calcium by frog heart muscle. Cosmos and Harris (12) demonstrated a similar inhibitory effect of sodium on calcium uptake by frog skeletal muscle. An effect of calcium concentration on sodium transport by intestine was reported by Dumont et al. (13). Walser (14) suggested that a common carrier for sodium and calcium transport existed in the renal tubule since sodium loading increased calcium output in the urine. Competition between sodium and calcium for a transport mechanism was also suggested by studies of cation transport across the rabbit gallbladder wall (15).

Irrespective of the theoretical basis for the effect of sodium concentration on concentrative calcium transport by chick intestine in vitro, the low sodium medium provides a technique for determining response of chick intestine to vitamin D. The concentrative transport of calcium by

chick intestine in vitro can be demonstrated only in the ileum. In this respect the chick intestine resembles that of the golden hamster (3). The in vitro studies of intestinal permeability to calcium under conditions in which concentrative transport is inhibited by N-ethylmaleimide show that vitamin D increases the rate of diffusion of calcium across the intestinal wall in the chick just as has been demonstrated for rat intestine (6). In the rat intestine, however, the intestinal permeability to calcium under in vitro conditions is greatest in the duodenal segment, whereas the chick duodenum in vitro does not permit the transfer of calcium from mucosal to serosal surface. This segment of the chick intestine has a much thicker muscular layer than the corresponding segment of the rat intestine and this may make it unsuitable for in vitro studies in which ion transport across the entire thickness of the intestinal wall is determined. The in situ studies of calcium efflux from tied off loops of intestine indicate that the duodenum is the site of the most rapid transfer of calcium out of the intestinal lumen and that this transfer of calcium is greatly enhanced by vitamin D.

ACKNOWLEDGMENT

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Effects of Dietary Elaidic Acid on Membrane Function in Rat Mitochondria and Erythrocytes [1]

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ABSTRACT Possible changes of membrane functions in rat erythrocytes and liver mitochondria due to incorporated elaidic acid were investigated. To this end, weanling rats were fed a diet containing 8% elaidinized olive oil which led to a significant incorporation of elaidic acid into the lipids of these structures. After 6 weeks, liver mitochondria from this group swelled at a rate 2 to 3 times greater than those from control rats fed native olive oil, when the experiments were performed in hypotonic medium containing inorganic phosphate. Rates of α-lecithinase-induced hemolysis were 5 times those of controls. Resistance of the red cells to osmotic stress, copper, chenodeoxycholate and non-ionic solutes were not much different in both groups. Transport of sodium ion proceeded at a slightly faster rate in red blood cells rich in elaidic acid.

In a previous publication (1), the incorporation of dietary elaidic acid into lipids of depot fat, erythrocyte membranes, and mitochondria was reported. The extent of incoporation of this *trans* fatty acid into the lipids of these tissues differed. Its influence on the fatty acid composition of the tissues was also different. Although the fatty acid composition of all tissues studied was significantly changed by the incorporated elaidic acid, that of depot lipid resembled the dietary fat more closely than did the lipid from mitochondria and red cell stromata. These results suggested a selective mechanism of incorporation of elaidic acid and raised the question whether the elevated *trans* fatty acid content would reflect itself in changes of membrane function. That dietary lipids influence various functions in both mitochondria (2) [3] and erythrocytes (3, 4) has been demonstrated, but little is known about the effect of *trans* fatty acids in these systems.

Therefore, the studies reported here were designed to measure some aspects of permeability and transport in isolated membranes from animals raised with and without dietary elaidic acid. Swelling rates of liver mitochondria were determined as a measure of permeability of these structures to water. Membrane functions of erythroctyes were studied by measuring simple diffusion of non-ionic solutes, active transport of sodium, and the relative stability of the membranes to various forms of hemolytic stress.

EXPERIMENTAL PROCEDURES

Male weanling rats, Walter Reed strain, were raised for 6 weeks with a 15% casein ration. The fat, supplying 8% of the diet, consisted either of native olive oil (controls) or of olive oil elaidinized by treatment with SO_2 (supplemented rats). This procedure produced a yield of 55% *trans* fatty acids. At the end of 6 weeks, during which growth rates and general well-being were not different in both groups, the animals were killed and their tissues used for analysis of fatty acid composition as well as for the studies presented here. The details of these procedures, of isolation of erythrocytes and mitochondria, as well as the analytical results, have been described (1).

Mitochondrial swelling. Mitochondrial swelling was measured by recording the decrease in absorbance at 520 mμ, using a Guilford Multiple Absorbance Recording

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Spectrophotometer. Cuvette temperature was maintained at 25°. The reaction mixture consisted of hypotonic swelling medium containing phosphate (5) 2 ml, or KCl-Tris (5), 2 ml; water, 0.8 ml; succinate or α-ketoglutarate (300 mM), 0.1 ml; NAD (3 μmoles) when α-ketoglutarate was the substrate, 0.1 ml; and a suspension of mitochondria obtained from 20 mg of liver in 0.1 ml. Results are expressed as change in absorbance units per minute during the initial linear portion of the curve.

Lecithinase-induced hemolysis. Nine and seven-tenths milligrams of bacterial α-toxin from type A *Clostridium perfrigens* [4] were diluted to 30 ml with isotonic saline. To 3 ml of this solution were added 5 μliters of fresh blood, and the decrease in absorbance at 520 mμ was followed, using the Gilford Spectrophotometer. Decrease in absorbance was taken as an index of hemolysis. Decrease due to settling of erythrocytes in isotonic saline was found to be less than 0.01 absorbance units in 10 minutes.

Sodium transport in erythrocytes. The procedure was a modification of that of Harris and Maizels (6). Fresh blood was centrifuged and washed twice with isotonic saline, 2 ml of packed cells were drawn off and added to 14 ml of incubation medium (300 ml of 0.15 M NaCl, 70 ml of 0.15 M KCl, 30 ml of 0.10 M Na_2HPO_4, and 4 ml of 50% (w/v) glucose; pH 7.7), to which 2.0 μCi $^{22}NaCl$ in isotonic saline had been added previously. This mixture was incubated in a Dubnoff metabolic shaker at 37° overnight to load the cells with ^{22}Na. The next morning, the suspension was centrifuged at 3200 × *g* for 10 minutes; the supernatant solution was discarded. The erythrocytes were washed once with 40 ml of isotonic glucose [5] and the cells were added to 100 ml of nonradioactive incubation medium. This mixture was shaken on the Dubnoff apparatus at 37°; at 0, 0.5, 1, 2, 3, and 4 hours, 10 ml of the suspension were withdrawn and placed into test tubes for radioactivity measurement. Ten milliliters of warm incubation medium were added back to the flask after each withdrawal to keep the pH constant and the external ^{22}Na in the medium at a negligible level.

The test tubes were centrifuged, the supernatant solution was decanted, and the erythrocytes were hemolyzed by the addition of 2 ml of distilled water. The hemolysates were assayed for radioactivity level in a Packard Auto-Gamma Spectrometer. Counts were corrected to the starting volumes of erythrocytes, and were expressed as a percentage of the original activity remaining. The logarithm of percentage activity remaining was plotted against time in hours to obtain the rate of sodium transport out of the erythrocytes.

Osmotic hemolysis. Resistance of the erythrocyte to osmotic hemolysis was determined by a modification of the method of Diggs (7). A series of 10 centrifuge tubes containing 4 ml of saline solution in concentrations of 0, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, and 1% received 7 μliters of fresh blood each. The tubes were shaken gently, allowed to stand for one hour at room temperature, and centrifuged at 3200 × *g* for 10 minutes. Supernatant solutions were decanted into a colorimeter tube and read at 415 mμ against a 1% NaCl blank in a Bausch and Lomb Spectronic 20 colorimeter. Hemolysis at each concentration of NaCl was calculated from absorbance using the following equation:

$$\% \text{ Hemolysis} = \frac{A_{\text{test solution}} - A_{1\% \text{ NaCl}}}{A_{0\% \text{ NaCl}} - A_{1\% \text{ NaCl}}} \times 100,$$

where *A* is the absorption of the supernatant of the blood samples subjected to the indicated treatments.

Chenodeoxycholate-induced hemolysis. Five microliters of fresh blood were added to 3 ml of a solution containing 1.0% sodium chenodeoxycholate in isotonic saline; decrease in absorbance was measured as in the lecithinase-induced hemolysis experiments.

Metal-induced hemolysis. A modification of the procedure of Lambin et al. (8) was used. Seven microliters of fresh blood were incubated in 4 ml of isotonic saline containing from zero to 7 μg of copper for one hour at room temperature. The tubes were processed as in the osmotic hemolysis

[4] Kindly provided by Mr. J. R. Evans, Department of Bacteriology, Walter Reed Army Institute of Research.

[5] Five per cent Dextrose Injection, U.S.P., Cutter Laboratories, Berkeley, California.

experiments, and the percentage hemolysis was calcualted:

$$\% \text{ Hemolysis} = \frac{A_{\text{test solution}} - A_{0 \text{ ug Cu}}}{A_{0\% \text{ NaCl}} - A_{1\% \text{ NaCl}}} \times 100,$$

where A is derived as in above equation.

Permeability of erythrocytes to nonionic solutes. Three milliliters of 0.3 M solutions of thiourea, ethylene glycol, or glycerol and 5 µliters of fresh blood were used to determine relative rates of permeability through relative rates of hemolysis as described in the lecithinase-induced hemolysis studies (9).[6]

RESULTS

In a hypotonic swelling medium containing inorganic phosphate, the liver mitochondria from elaidic acid-supplemented rats exhibited significantly greater initial rates of swelling than controls. The magnitude of the difference depended on the substrate added: With succinate, the difference was more than twofold (0.03 vs. 0.07 absorbance units/minute); with α-ketoglutarate and NAD, it was more than threefold (0.05 vs. 0.17 absorbance units/minute) (fig. 1). In KCl-Tris medium, mitochondria from the two dietary groups behaved alike, regardless of substrate used.

A highly significant difference between the two dietary groups was noted when red blood cells were incubated with lecithinase (fig. 2). After a lag period of 2 minutes, erythrocytes containing *trans* fatty acids hemolyzed at a rate 5 times faster than that of the control cells (0.15 vs. 0.03 absorbance units/minute). After 12 minutes, the rates of both groups were approximately 0.03 units/minute; after 25 minutes they slowed markedly and came to a standstill at approximately 2 hours. Hemolysis apparently was complete at that time.

[6] The animals used in this study were treated in accordance with the principles of laboratory animal care as promulgated by the National Society for Medical Research. The mentioning of trade names and manufacturers does not constitute an official endorsement of these products by the U.S. Government.

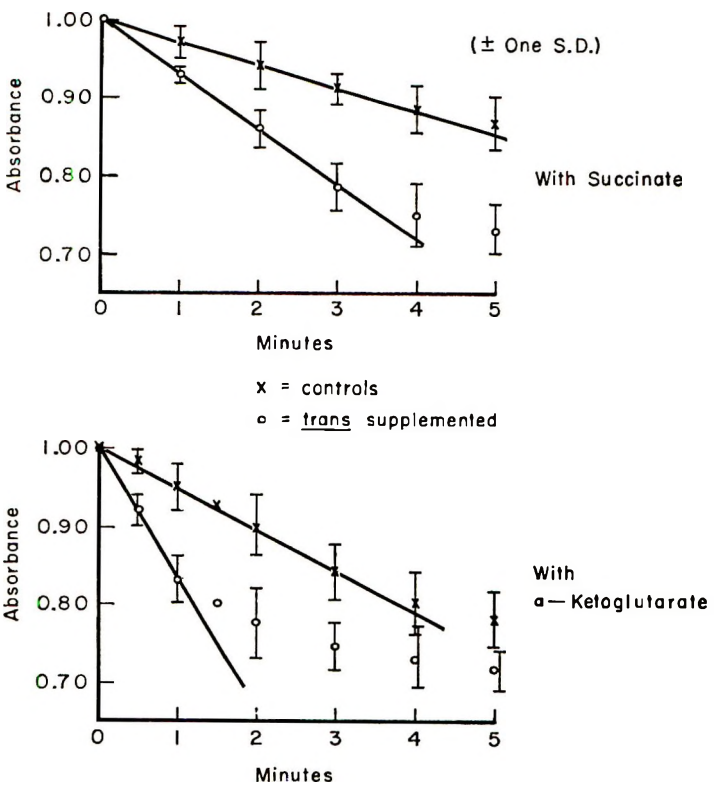

Fig. 1 Swelling of liver mitochondria in hypotonic medium (N = 3).

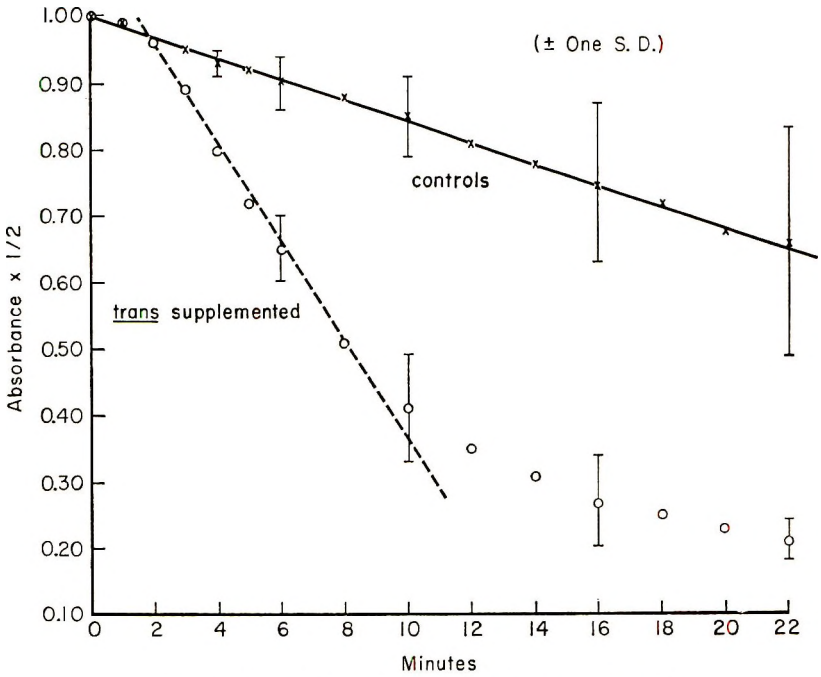

Fig. 2 Hemolysis induced by lecithinase (N = 3).

Compared with these results, the differences between the two dietary groups in the following experiments were much smaller. The erythrocytes of rats fed the elaidic acid-supplemented diet transported sodium ion at a slightly faster rate than those of the controls. The plot of logarithm of the percentage of initial radioactivity remaining in the cells, against time, resulted in straight lines for each group (fig. 3). Both linear regressions of each rate as well as the difference between the rates were significant ($P < 0.01$ and < 0.05, respectively.

The results of experiments measuring the effect of various hemolytic agents on red blood cells are summarized in table 1 and 2. The effects of copper, diethylene glycol and thiourea were nearly identical in the erythrocytes from both groups. The cells from elaidic acid-supplemented rats appeared somewhat more resistant to osmotic stress and to chenodeoxycholate, and less resistant to glycerol than their controls.

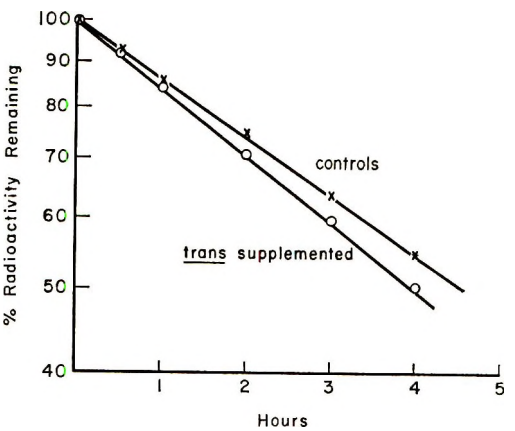

Fig. 3 Sodium extrusion of rat erythrocytes (N = 5).

DISCUSSION

The data presented here indicate that the *trans* fatty acid incorporated into mitochondria and red cell membrane affects a number of functions related to permeability and transport; the magnitude and direction of these changes depended on the systems studied. The increase of the swelling rate in mitochondria from elaidic acid-supplemented rats is striking, as is the faster rate of lecithinase-induced hemolysis in erythrocytes from these animals.

TABLE 1

Effect of hemolytic agents on rat erythrocytes

Concentration		Hemolysis	
NaCl	Cu^{++}	Control	Elaidic acid-supplemented
%	μg	%	%
		Osmotic hemolysis [1]	
0.45		61.4 ± 8.9 [2]	57.9 ± 7.1
0.40		86.2 ± 6.7	80.8 ± 6.5
0.30		95.0 ± 2.9	90.4 ± 3.9 [3]
		Copper-induced hemolysis [4]	
	0	0	0
	1	22 ± 4	21 ± 3
	2	23 ± 2	22 ± 2
	3	29 ± 4	29 ± 5
	4	42 ± 2	47 ± 4
	5	63 ± 6	63 ± 6
	6	75 ± 4	71 ± 2
	7	91 ± 8	89 ± 3

[1] Controls, N = 10; elaidic acid supplemented, N = 11.
[2] Mean ± SD.
[3] Difference between 2 groups significant ($P < 0.05$).
[4] N = 6 in both categories.

A number of other stresses, nevertheless, produced nearly identical results in both groups, and it is not clear whether some of the small differences, although statistically significant, have physiological importance. These varied reactions, dependent on the particular stress applied, offer a tentative delineation of the sites in the red cell membrane and mitochondrion which are affected by the incorporated *trans* isomer.

One such site can be discussed in more detail. Since nearly all of the mitochondrial lipid is part of the membrane (10), it appears reasonable to assume that the effect of dietary elaidic acid is due to its incorporation into the mitochondrial membrane. Of the various substances which induce swelling, the endogenous uncoupling factor, or U-factor (11) is of particular interest in relation to our results. It has been described as a mixture of higher fatty acids, released through enzymatic action from membrane phospholipids, and is responsible for uncoupling of oxidative phosphorylation and swelling (11). It is possible that membrane phospholipids containing elaidic acid are more susceptible to enzymatic hydrolysis than those of control animals. This possibility is supported by the results of experiments measuring hemolysis induced by α-lecithin-

TABLE 2

Effect of hemolytic agents on rates of hemolysis; change of absorbance units/minute [1]

Compound	Control erythrocytes			Elaidic acid-supplemtned erythrocytes		
	Initial	Secondary	Tertiary [2]	Initial	Secondary	Tertiary
Chenodeoxycholate	2.16 [3] ± 0.12	3.50 [4] ± 0.07	0.02 ± 0.01	1.98 [3] ± 0.02	3.20 [4] ± 0.07	0.02 ± 0.01
Glycerol	0.70 [3] ± 0.04			0.82 [3] ± 0.04		
Diethylene glycol	0.86 ± 0.15			0.87 ± 0.02		
Thiourea	0.84 ± 0.04	2.93 ± 0.03	0.063 ± 0.012	0.81 ± 0.04	2.93 ± 0.23	0.063 ± 0.016

[1] Each value represents mean ± SD of 6 rats.
[2] Indicating initial, secondary and tertiary portion of sigmoid curve.
[3] Difference between means of experimental animals and controls is significant; $P < 0.05$.
[4] $P < 0.01$.

ase. Since it has been shown that hemolysis is proportional to the rate of hydrolysis by the enzyme (12), the faster rate in the cells high in elaidic acid was probably the result of greater susceptibility of the lecithins containing the *trans* fatty acid to enzymatic attack. The position of elaidic acid in a phospholipid molecule has not yet been defined.

It is not clear why the difference in swelling between mitochondria containing elaidic acid and controls was observed only in hypotonic swelling medium, and not in KCl-Tris. The former medium contains inorganic phosphate which, by itself, induces a measured amount of swelling (5), and it is possible that this may be a permissive factor to reveal the differences between the mitochondria from the two dietary groups. The dependence of swelling rates on the exogenous substrate in our experiments confirms previous observations (5).

The difference in rates of sodium transport between the erythrocytes from the elaidic acid-supplmented group and controls is small, and it is questionable whether any physiological significance can be attributed to these results. However, the fact that 2 groups of phospholipids, phosphatidic acid (13) and inositides (14) have been cited as specific carriers in sodium transport, offers a possible explanation of the observed effects, in the light of our previous observations with lecithinase-induced hemolysis. The latter suggest that the incorporation of elaidic acid imparts a different property to the lecithin, and it appears possible that similar differences may exist for phospholipids involved in sodium transport, making them slightly more efficient as carriers. The absence of great differences between the dietary groups in the other experiments measuring hemolysis allows the conclusion that the membrane sites susceptible to the attack of hypotonic solutions, of chenodeoxycholate, of nonionic solutes and of copper were not much affected by elaidic acid.

To attribute the observed effects of dietary elaidic acid to its incorporation into membrane phospholipids appears to be a reasonable hypothesis, but a second possibility must also be considered. The feeding of the *trans* fatty acid produced a change of lipid composition also with respect to other fatty acids (1). Particularly, the relative concentrations of linoleic and arachidonic acids in the elaidic acid-supplemented mitochondria and stromata were considerably less than those of controls. It is known that dietary restriction of unsaturated *cis* fatty acids alone can alter mitochondrial stability,[7] but the degree of these changes is far less than the degrees observed in the present experiments. Variations in the concentration of oleic and other unsaturated fatty acids have been linked with large changes in the permeability of red blood cells to glycerol (15), which, in our studies, was little affected. From these considerations, it appears likely that the observed effects were caused directly by the elaidic acid in the tissues and not indirectly by its effect on the tissue distribution of other fatty acids.

The results presented here do not allow a conclusion as to the nutritional significance of elaidic acid for membrane functions, since it was impossible to obtain tissues free of the *trans* isomer (1). The study compared animals rich in elaidic acid with controls containing a small concentration, which may have been sufficient for maintenance of a near-normal function. However, the fact that small concentrations of the *trans* fatty acid were consistently noted in the tissues, but not in the diet of the control animals (1), and the differentiated influence of dietary elaidic acid on various aspects of permeability suggest that this acid may play a physiological role in the function of membranes.[8]

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Fatty Liver in the Rat after Prolonged Intake of Ethanol with a Nutritionally Adequate New Liquid Diet [1,2]

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ABSTRACT To determine whether prolonged ethanol intake can produce a fatty liver, even when associated with a diet containing adequate amounts of protein, minerals and vitamins, rats were given liquid diets containing 18% of calories as casein, supplemented with methionine (0.3 mg/kcal) and cystine (0.5 mg/kcal), choline (0.25 mg/kcal), fat (35% of total calories), adequate minerals and vitamins and, in the control diet, 47% of the calories as carbohydrates. A littermate of each control rat was pair-fed with the same diet in which carbohydrates had been isocalorically replaced with alcohol to the extent of 36% of the total calories. These diets assured continued growth in all animals and normal liver in the controls, whereas in the rats fed with alcohol, fatty liver developed, which was evident both morphologically and on chemical analysis; after 24 days of alcohol, hepatic triglycerides had increased on the average sixfold and cholesterol esters fivefold, compared with those of the controls. These studies demonstrate that prolonged alcohol intake can produce a fatty liver even when given with a diet with nutritionally adequate content of protein, vitamins and minerals, and an amount of fat less than that of the average American diet.

There is a widespread belief that when alcohol is ingested with an adequate diet, it produces no liver damage. This concept was challenged when we showed previously that both in man and in rats, fatty liver could be produced by prolonged alcohol intake despite diets with adequate content in nutrients (1, 2). To overcome the natural aversion of rats for alcohol, totally liquid diets, containing in one formula the necessary nutrients, as well as alcohol, were used. Our former purified diet contained a complete amino acid mixture as a substitute for protein, sucrose as carbohydrate and an amount of fat comparable to that of an average American diet (43% of total calories). With such a diet, isocaloric substitution of carbohydrates with ethanol to the extent of 36% of total calories resulted, after 24 days, in an average 7- to 8-fold increase of hepatic triglycerides (1, 2). The present study was undertaken to determine whether a similar ethanol effect could be demonstrated with a diet containing, instead of amino acids, a protein (casein), and instead of sucrose, a dextrin-maltose mixture which more closely resembles carbohydrates commonly found in food. The fat content of the diet was also reduced to 35% of the calories.

MATERIALS AND METHODS

Male rats of a Sprague-Dawley strain (CD) were used in 11 groups of 2 littermates each. They were maintained with a commercial laboratory ration [4] and tap water ad libitum until they had reached a weight of 100 to 150 g, at which time they were housed in individual wire-bottom cages and given liquid diets in drinking tubes as the only source of food and water.

The overall composition of the new diet is shown in figure 1, together with our previous formula, to facilitate comparison of the changes made. The composition of the ethanol and control diets was as follows: casein [5] (supplemented with methionine 0.3 mg/kcal, and cystine 0.5 mg/kcal), 18% of the total calories; fat, 35% of total calories; adequate vitamins and minerals;

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[3] Dr. Lieber is recipient of Public Health Service Research Career Development Award K3-AM22,590 from the National Institute of Arthritis and Metabolic Diseases.

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

[5] Vitamin-free Micro pulverized Casein, Nutritional Biochemicals Corporation, Cleveland.

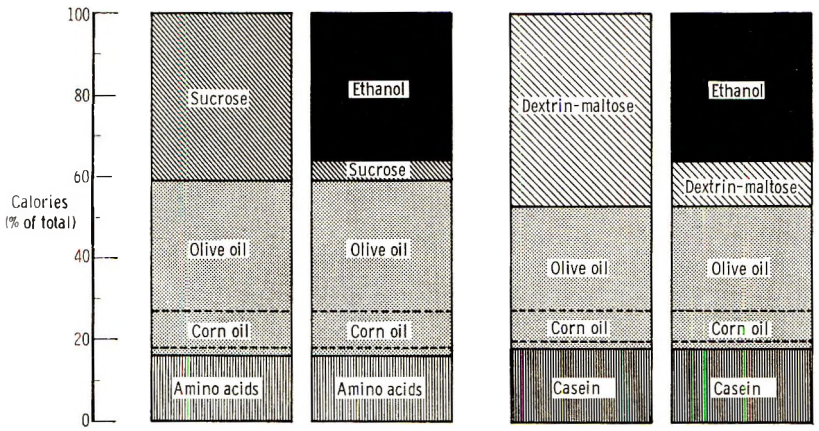

Fig. 1 Composition of liquid diets fed to the rats; amino acid, vitamin and mineral content was as described previously (22). Gray section below corn oil: ethyl-linoleate, 2 mg/kcal. Casein supplemented with methionine, 0.3 mg/kcal and cystine, 0.5 mg/kcal.

and in the control diet, carbohydrate,[6] 47% of the total calories. In the ethanol formula, carbohydrate was isocalorically replaced to the extent of 36% of the total calories.

The choline content and the calculated methionine composition were 0.25 and 1.5 mg/kcal, respectively, which is equivalent to concentrations of 0.1 and 0.6% in solid diets, amounts found to be optimal for the rat (3). The diets contained 1 kcal/ml, an adequate water-to-calorie ratio for the rat (4). Sodium carrageenate[7] (2 g/liter) was used to stabilize the liquid diet.

Animals were fed in groups of 2 littermates each: one rat was given the control diet, while the other received the same regimen in which dextrin-maltose, to the extent of 36% of calories, was isocalorically replaced with ethanol. Loss of ethanol by evaporation was negligible in the diets kept in the graduated drinking tubes (5) for periods of up to 48 hours. The diet was changed every 24 hours and, in some experiments, every 12 hours. Ethanol was introduced into the diet gradually. The final concentration of 50 g/liter of ethanol was achieved on the fifth day of feeding. During the first 2 days, the animals were given the liquid diet with 30 g/liter ethanol which was increased to 40 g/liter for the third and fourth days. Observation during the initial days indicated in each pair of littermates which of the animals had the lowest spontaneous food intake. This rate-limiting rat (which was usually the one given ethanol) received the liquid diet ad libitum and the corresponding littermate was fed isocaloric amounts of the other diet on the following day. During the 24 hours preceding killing of the rats, the diets were given in three divided doses at approximately 8-hour intervals.

The body weight of the rats was determined at least twice a week. After 24 days, the animals were decapitated and blood was collected from the neck vessels in heparinized tubes and plasma was obtained by immediate centrifugation in the cold. The liver was quickly excised and approximately one gram was weighed into tubes containing chloroform:methanol (2:1). The plasma and remaining liver were stored at −18°.

Total hepatic lipids were extracted (6) and quantitated by the method of Amenta (7). An aliquot of the total lipid extract, containing approximately 20 mg of fat, was evaporated under nitrogen to a volume of about 0.5 ml, and applied to a 0.5-mm thick silica gel chromatoplate (8) and developed in hexane:diethyl ether:acetic acid (83:16:1). The triglycerides, cholesterol esters and free cholesterol were eluted by the method of Goldrick and Hirsch (9); triglycerides were quantitated by determi-

[6] Dexin, generously supplied by Dr. Singleton, Burroughs Wellcome and Company, Tuckahoe, New York.
[7] Viscarin, Marine Colloids, Inc., P.O. Box 70, Springfield, New Jersey.

nation of ester linkages by the procedure of Snyder and Stephens (10), and cholesterol and cholesterol esters were determined by the procedure of Searcy and Bergquist (11). Plasma alcohol was determined by the method of Bonnichsen (12). Samples of hepatic tissue were fixed in 10% neutral formalin until they were processed for histological examination.

The results obtained from each animal were compared with the corresponding values in the pair-fed control littermate. The means (± SE) and individual differences were calculated and the degree of significance was determined by Student's *t* test (13).

RESULTS

Twenty-four days of isocaloric replacement of carbohydrate by ethanol resulted in a significant increase of total hepatic lipids to 96.8 ± 6.6 mg/g, compared with only 46.1 ± 1.3 mg/g ($P < 0.001$) in the controls. This increase in hepatic total lipids resulted primarily from a change in hepatic triglycerides and cholesterol esters as indicated in figures 2 and 3, respectively. The average triglyceride increase was almost sixfold, from 10.8 ± 0.64 to 56.8 ± 4.6 mg/g, whereas the average increase in cholesterol esters was fivefold, from 0.59 ± 0.05 to 2.92 ± 0.14 mg/g. Free cholesterol changed only slightly as indicated in figure 3.

Alcohol levels, determined on the plasma taken at the time of killing, varied

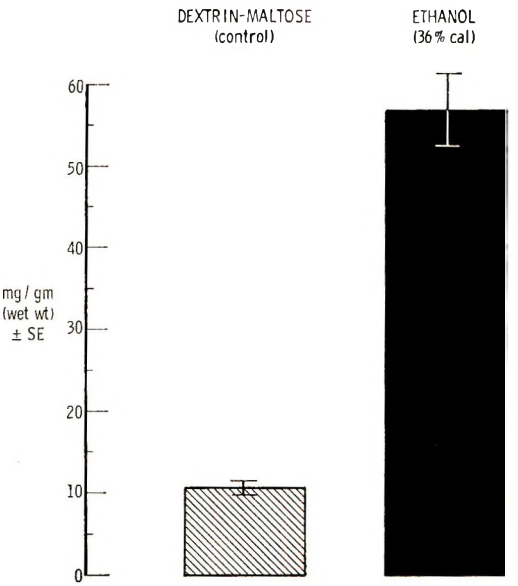

Fig. 2 Hepatic triglyceride concentration in rats pair-fed for 24 days with liquid diets containing either dextrin-maltose (control) or isocaloric amounts of ethanol (36% of calories).

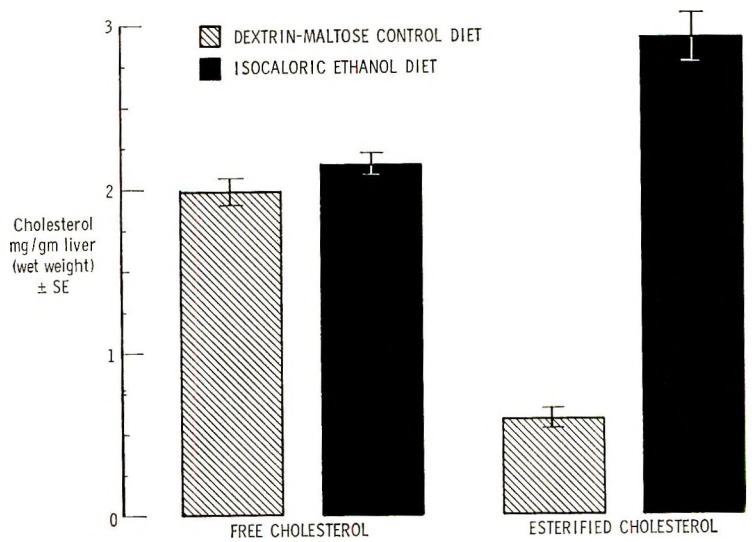

Fig. 3 Hepatic cholesterol concentration in rats pair-fed for 24 days with liquid diets containing either dextrin-maltose (control) or isocaloric amounts of ethanol (36% of calories).

widely with a mean of 145.1 ± 23.7 mg/100 ml.

With both diets, rats continued to grow, with an average daily weight gain of 3.05 g.

With hematoxylin and eosin stains, hepatic morphology was found to be normal in controls, whereas fat accumulation was evident in the rats given alcohol, substantiating the chemical determinations.

DISCUSSION

The purpose of the present paper was to determine whether prolonged ethanol intake can result in fatty liver, despite a diet adequate in protein, vitamins and minerals. With conventional feeding techniques, that is, incorporation of alcohol in drinking water and administration of solid food, rats limited their alcohol intake, in most studies, to 20% of total calories or even less (14, 15); only in one study was the intake as high as 30% of total calories (16). With these techniques, however, as long as the dietary intake was adequate, no pronounced accumulation of fat in the liver was detected (14–16). This lack of steatosis is not unexpected, since 20% of total caloric intake as alcohol is a very low dose which, in our laboratory, not only failed to produce an appreciable fatty liver, but also did not result in any significant blood level of alcohol (2). No blood alcohol levels were reported in the one study in which 30% of total calories as alcohol failed to produce a fatty liver (16). To determine whether hepatic steatosis can be produced in rats with ethanol in the absence of dietary deficiency, an experimental method was needed to increase the amount of alcohol consumed by the rats. Alcohol, when given acutely without food in a large single dose by gastric tube, was found to produce fat accumulation in the liver (17–22) but the mechanisms involved in these acute experiments do not necessarily apply to more prolonged alcohol intake such as observed in chronic alcoholic patients (2, 22, 23). In our previous studies (1, 2, 24), prolonged intake of substantial amounts of alcohol was achieved by overcoming the natural aversion of rats for alcohol by incorporation of the ethanol in a completely liquid diet. This previous diet, however, contained, as a substitute for protein, an expensive amino acid mixture. The present study demonstrates that a similar effect can be obtained with alcohol when the amino acids are replaced by casein, enriched with methionine and cystine. To eliminate possible direct effects of sucrose on hepatic lipid metabolism which have been described by some (25, 26) but not observed by others (27), sucrose was replaced by a mixture of dextrin-maltose. The diet was further changed by decreasing its fat content from 43 to 35% of total calories, an amount less than that of the average American diet (28). This decrease in fat content allowed an increase of the carbohydrate from 41 to 47% in the control diet; in the ethanol diet this resulted in a doubling of the remaining carbohydrate after incorporation of the ethanol. In addition to a composition in all known required nutrients in amounts considered to be adequate or optimal for the rat (29), the quality of the diet was evidenced by the continued growth of the rats as well as normal hepatic fat content and morphology in the controls.

Lipotrope content of our diet (0.25 mg of choline and 1.5 mg of methionine/kcal, including the methionine present in casein) was equivalent to the amount of lipotropic substances reported by Klatskin et al. (30) and Best et al. (31) to fully protect against fatty liver development in rats given a choline-deficient diet, with or without ethanol in drinking water. The amount of choline was also reported by others to be optimal for the rat (3). Since the possibility has been raised that ethanol may increase choline requirements (30), and since the ethanol intake in the present experiments was greater than that in the studies of Klatskin et al. (30), the possibility has to be considered that choline requirements may have been increased even further. It is unlikely, however, that simple enhancement of choline requirements could fully explain our results, since in one of our previous studies, massive choline supplementation with 20 times the amount present in our diet failed to fully protect against steatosis; hepatic triglyceride accumulation, although reduced, still represented a threefold increase compared with the controls (24). It is therefore likely

that ethanol produces steatosis through effects other than or in addition to those related to lipotrope metabolism. The present study demonstrates that this hepatic steatosis can be produced by ethanol even when our original formula (1, 2, 24) is modified to more closely resemble conventional diets by replacing sucrose by dextrin-maltose, amino acids by protein, and by decreasing the fat content to an amount less than that of the average American diet. This improved procedure for the experimental production of a fatty liver on prolonged alcohol ingestion is proposed as a convenient and inexpensive tool for further studies of the pathogenesis and possibly the treatment and prevention of alcoholic liver disease.

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Estimation of Methionine Synthesis in Intact Cows after Administering Sulfide-^{35}S [1,2]

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ABSTRACT A study was made to determine the amount of methionine synthesized in the rumen. Sodium or barium sulfide-^{35}S was given orally as a source of sulfur for ruminal methionine synthesis in cows adjusted to controlled levels of constant feed intake. The 24-hour turnover of methionine was determined by adding to the daily rations increments of fish meal, a protein source found to be 91% undegraded in passing through the rumen. The logarithm of the decline in specific activity of methionine in milk protein closely fitted a linear regression indicating that methionine was behaving as a single pool undergoing simple dilution. It was therefore possible to estimate the pool size of the net methionine available for use in milk synthesis. Net methionine available was resolved into synthesized and food methionine by regression procedures. Daily methionine synthesis in cows eating alfalfa varied between 31 and 59 mg/kg of body weight.

This study is concerned with the biosynthesis of methionine in the rumen and its transfer to milk proteins.

Most nitrogen compounds entering the digestive tract of cattle are degraded to ammonia totally or in part depending on their solubility in the rumen. In turn the ammoniacal nitrogen becomes the precursor to the nitrogen of amino acids biosynthesized during microbial growth. After protein digestion in the gastrointestinal tract, amino acids arising from the microbial protein can enter the circulatory system and tissue fluids and be used subsequently for assimilation or in the intermediary metabolism of cow's cells (1). Loosli et al. (2) showed that ten essential amino acids for rat growth were synthesized in the rumen of sheep and goats. Black et al. (3) observed that aside from the alimentary synthesis by rumen microorganisms, cows require the same amino acids which are essential for the rat. These investigators bypassed the rumen and its population of rumen microorganisms by injecting ^{14}C-labeled acetate, carbonate, butyrate and propionate intravenously. It was then possible to distinguish between amino caids originating exogenously and the amino acids synthesized in quantity by the cow's tissue.

A corollary to these observations is that a particular essential amino acid may be labeled during ruminal synthesis by introducing into the rumen a precursory radioactive compound specific for the amino acid. If steady-state conditions prevail in the rumen with respect to microbial growth, the relative amount of labeled precursor incorporated into the amino acid is proportional to the total amount of the amino acid that is produced.

The relative amount of labeled amino acid assimilated in the milk proteins provides a sampling system. The specific activity of a milk amino acid so labeled is useful for describing the kinetics and quantitating the turnover of the amino acid. Of various approaches tried, we have had the most success with a procedure that utilized a single dose of ^{35}S-labeled sodium or barium sulfide as a precursor to methionine sulfur in cows consuming feed at a uniform rate.

METHODS

An experiment with an individual cow included three consecutive 3-day periods. Sodium sulfide-^{35}S or barium sulfide-^{35}S was given orally at the beginning of each

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3-day trial. One to five millicuries of ^{35}S were used. A steady state was maintained in the cows by feeding at 4-hour intervals during the first 36 hours of a trial and then at 12-hour intervals for the remaining 36 hours. The cows were also milked according to this time schedule.

In the first period no supplemental dietary methionine was added. In the second period supplemental methionine was added in the form of fish meal. Fish meal was selected because it was known to be slowly degraded in the rumen and is relatively high in methionine (4, 5). In the third trial the amount of fish meal methionine fed was twice that used in trial 2. To estimate the amount of methionine degraded in the rumen, two 24-hour digestion studies in vivo were carried out in the rumen of a fistulated steer, using a Dacron bag technique described by Hopson et al. (6) and modified to include fish meal. The mean values used in estimating ruminal degradation of methionine are presented in table 1. In separate studies the half-time for disappearance of fish meal protein placed directly in the rumen and mixed with the contents was found to be 8.6 hours. On the basis of disappearance and degradation rates it was estimated that 90.8% of the fish meal methionine passed out of the rumen undegraded.

Experiments involving 3 trials were carried out with 2 cows fed each of 2 lots of alfalfa. The alfalfa was harvested in the bud stage. The first lot of alfalfa was artificially dried and preserved as chopped hay. It contained 3.30% nitrogen in the dry matter. The second lot of alfalfa had been harvested as silage at 32% dry matter. It contained 3.01% nitrogen in the dry matter.

The milk proteins were separated from skim milk by acid precipitation, washed, solubilized for counting in a liquid scintillation counter or hydrolyzed for amino acid analyses. Casein was precipitated from skim milk with an acetate-acetic acid buffer at pH 4.45.

RESULTS

Milk specific activity. The radioactive methionine pattern found in milk protein was similar to that reported by Block et al. (7). Labeling of methionine in total protein tended to lag behind the labeling of casein (fig. 1). For this reason, casein was chosen as the preferred protein fraction for sampling methionine labeling of the nutrient pool. The specific activity of the milk protein presumably reflects the summation of the partial labeling pattern of proteins arising from several sites of

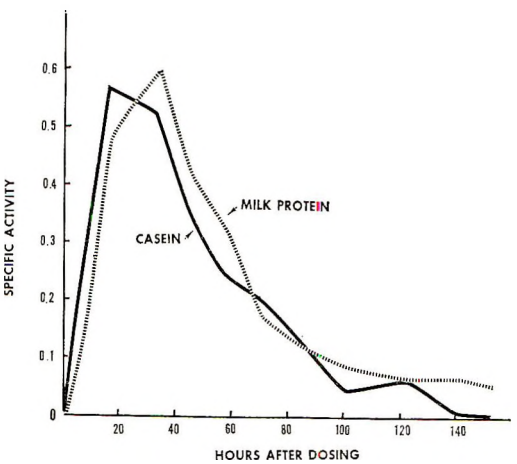

Fig. 1 Changes in the specific activity of total milk protein and casein with time after administering an oral dose of barium sulfide-^{35}S.

TABLE 1

Digestion of fish meal methionine from Dacron bags suspended in the rumen

Time	Crude protein	Methionine	Methionine remaining
hr	*mg*	*mg*	%
0.0	298	3.82	100
0.5	272	3.60 ± 0.02 [1]	94.5
2.0	268	3.56 ± 0.03	92.9
6.0	266	3.52 ± 0.05	92.1
12.0	252	3.34 ± 0.01	87.5
24.0	246	3.26 ± 0.02	86.5

[1] SD.

synthesis. The changes in specific activity for successive trials with time after feeding labeled sulfide are typified by response curves presented in figure 2. Although the increase in specific activity during the early hours is not linear, efforts to improve the fit of a regression line by several methods of rectifying the data failed to reduce the variability.

A semilogarithmic plot of the specific activity of casein methionine regressed on time is shown in figure 3. Directly beyond the maximal values, specific activity decreased exponentially with time; the individual values fitting well the least squares linear regression. This result was interpreted to mean that, once the effects of incorporating ^{35}S into microbial methionine became nil, entrance of methionine into the mammary pool proceeded kinetically as a single pool undergoing simple dilution.

Nutrient pool. The nutrient pool was designated as the total amount of methionine arising from protein digestion and tissue catabolism which enters the general circulation. Its size is proportional to the reciprocal of the specific activity. Theoretical content of the nutrient pool was calculated by regressing the reciprocal of the mean specific activity for two turnover times during the 3-day trials on the amount of methionine in the fish meal added in the diet during that time. The pool size is the regression constant for the intercept at zero methionine divided by the regression coefficient. It may also be found geometrically by extrapolating the regression line to the point where the reciprocal of the specific activity approaches

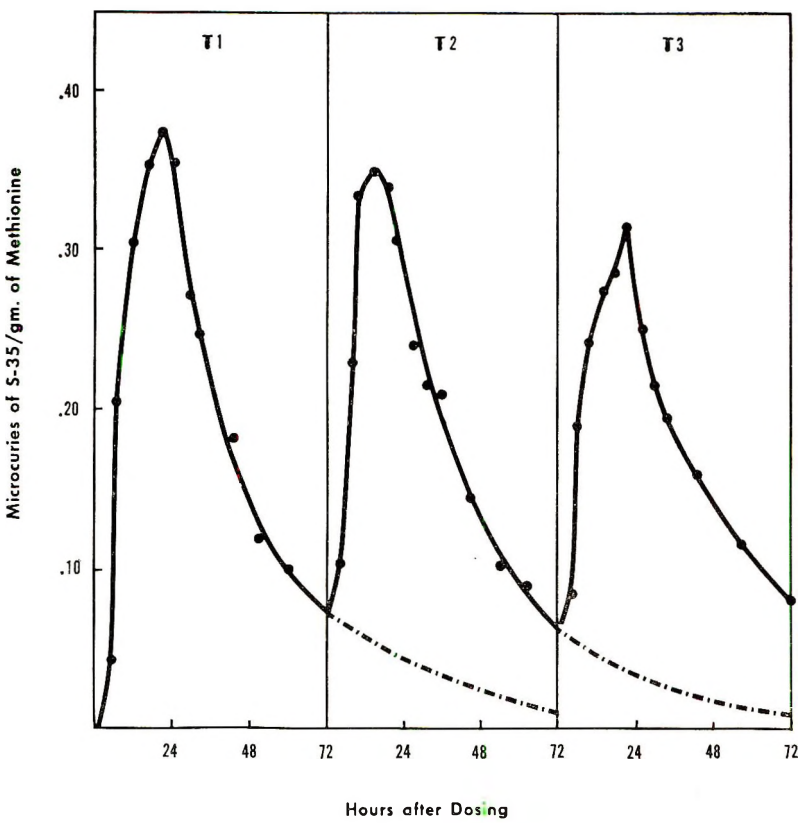

Fig. 2 Radioactivity curve of ^{35}S in milk methionine during 72-hour periods after dosing. T1, no methionine added; T2, 8.5 g of methionine added in fish meal; and T3, 17.0 g of methionine added in fish meal.

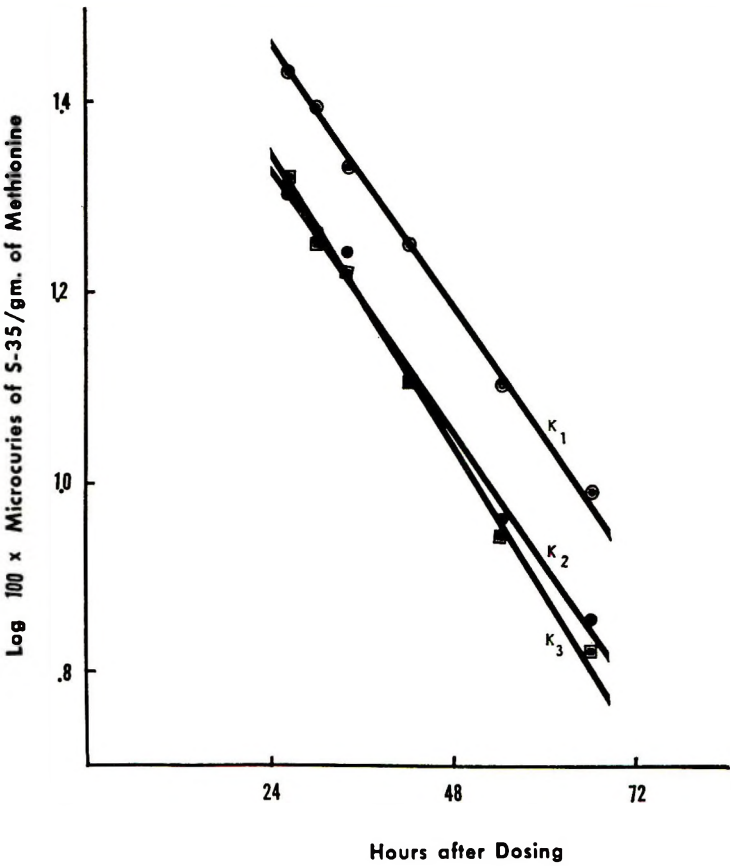

Fig. 3 Semilogarithmic plot of descending slope of radioactivity in milk methionine. K_1, no methionine added; K_2, 8.5 g of methionine added in fish meal; and K_3, 17.0 g of methionine added in fish meal.

zero as indicated graphically in figure 4. Mean values for the nutrient pool are presented in table 2.

Individual values for the pool size in each trial were obtained by solving the equation:

$$M_1 = \frac{\overline{M}s_1}{\overline{s}}$$

where M_1 was the pool size in grams, $\overline{M}$ was the mean size of the 3 pools. The $\overline{s}$ was the mean specific activity of the 3-pool composite and s_1 was the mean specific activity of the methionine in a particular trial. In these calculations it was assumed that the changes in radioactive sulfur in the methionine of casein was proportional to changes in the amount of ruminally synthesized methionine entering the nutrient pool.

The slope (k) of the declining regression line (fig. 3) multiplied by 2.3 to convert to natural logarithms represents the turnover rate (r) of the milk methionine pool. The milk methionine pool size was then obtained by multiplying the methionine flux by the turnover time, $1/r$. The turnover time of the milk methionine pool was assumed to be equivalent to the turnover time of methionine in the nutrient pool.

The daily methionine turnover (table 2) in terms of grams per day, was calculated from the nutrient pool and was therefore biased to the extent that there may have been net gains in methionine which arose from the tissue protein pool. Partitioning into the amounts attributable to microbial synthesis and the amount derived directly

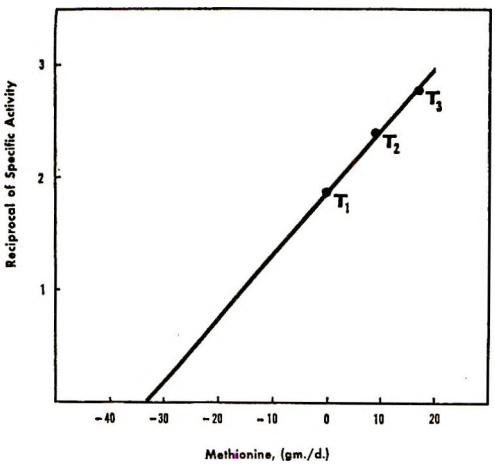

Fig. 4 Extrapolation of reciprocal of the specific activity of the milk methionine to zero was used to estimate the nutrient pool. T_1, no methionine added; T_2, 8.5 g of methionine added in fish meal; and T_3, 17.0 g of methionine added in fish meal.

from abomasal digestion of food protein was accomplished by statistical procedures. The total pool size for each of the 3 trials was regressed on the microcuries of ^{35}S contained in the pool. The synthesized methionine was estimated by multiplying the regression slope, change in pool size per microcurie of change in radioactivity of nutrient pool, by the total microcuries determined for the nutrient pool. Feed methionine was calculated as the difference obtained by subtracting the synthesized methionine from the total methionine in the nutrient pool.

Results of replications for 2 cows consuming alfalfa are presented in table 2. The amount of methionine synthesized conforms to theoretical expectations. Repeatability of means of replicates indicates that reliable estimates were made with this method. Large sample (within trial) standard errors in the first replicate of alfalfa 1 and the second replicate of alfalfa 2 are caused partly by bias from differences between regression points which arose from the variable turnover times determined for the radioactive methionine.

The usefulness of this method for estimating methionine synthesis was considered to depend upon the validity of 3 assumptions. They were 1) that, in cows fed at constant levels of feed intake, the contribution of undegraded feed methionine and net gains in methionine from the body protein pool remained relatively constant. (The corollary to this is that deviations in the radioactive sulfur content of casein were in general proportional to the amount of synthesized methionine entering the nutrient pool); 2) that equivalent percentages of fish meal protein and microbial protein were absorbed from the intestinal tract; and 3) that the degradation of methionine was estimated correctly.

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TABLE 2

Replications of turnover times and estimates of synthesized methionine in the cow's nutrient pool [1]

	Dried alfalfa		Ensiled alfalfa 2	
	Replication no.		Replication no.	
	1	2	1	2
Dry matter intake, kg/day	8.92	8.76	6.98	7.75
Nitrogen intake, g/day	287	297	204	229
Turnover time, hr	30.6 ± 12.4 [2]	31.1 ± 1.1	48.9 ± 5.7	32.3 ± 3.5
Nutrient pool, g	53.8	46.5	49.4	29.2
Methionine turnover, g/day	46.6	35.8	24.4	22.8
Undegraded methionine, g/day	23.2 ± 2.8	14.8 ± 0.4	10.1 ± 0.2	8.4 ± 1.3
Synthesized methionine, g/day	23.4 ± 3.8	21.0 ± 0.7	14.3 ± 0.2	14.4 ± 2.2
Synthesized methionine, g/kg dry matter	2.66	2.39	2.05	1.82
Synthesized methionine, mg/kg/day	59.2	54.4	35.2	31.3

[1] Three trials in each replication.
[2] SE.

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Effect of Plane of Nutrition and Source of Nitrogen on Methionine Synthesis in Cows [1,2]

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ABSTRACT A study was made to evaluate the effects of level of feed intake, ration composition and source of nitrogen on the synthesis of methionine in the rumen. The most significant effects on ruminal synthesis of essential amino acids were level of dry matter intake and level of nitrogen intake. Mean daily ruminal synthesis of methionine ranged between 32.8 and 46.2 mg/kg of body weight. The step-by-step insertion of the variables, kilograms of dry matter, grams of alfalfa nitrogen, percentage nitrogen absorbed and treatment of alfalfa, into a multiple regression analysis accounted for 94% of the total variation. It was concluded that methionine synthesis increased at a rate of 1.5 g/kg of feed consumed and 6.7 g/100 g of alfalfa nitrogen eaten. A marked decrease in methionine synthesis was found to be related to reduced nitrogen absorption in the digestive tract. Synthesis of methionine increased as the integrity of plant proteins was preserved and soluble nonprotein nitrogen decreased correspondingly. Methionine synthesis increased 0.4 g/kg of intact protein fed.

The objective of this study was to quantitate the effects of dietary factors on the synthesis of methionine in the rumen of lactating cows. Specific factors studied were plane of nutrition, composition of the ration, and the chemical form of nitrogenous compounds.

In an earlier study (1) the 24-hour turnover of methionine was found to range between 23 g/day and 47 g/day. Among individual cows, synthesized methionine ranged between 31 mg and 59 mg/kg body weight per day.

Indirect evidence obtained in balance studies and feeding trials has led to the conclusion that the requirement of the lactating cow for essential amino acids is met through the synthesis of microbial protein in the rumen (2–4). Subsequently more direct measures were made with ruminally synthesized protein labeled with ^{35}S and ^{15}N (5, 6). In an elaborate study of the nitrogen metabolism of dairy cows, Virtanen (7) observed the plasma levels of histidine and methionine to be relatively low in cows fed a purified ration containing only nonprotein nitrogen. The studies with labeled sulfur and nitrogen were made on one and two cows, respectively, and consequently are inadequate for deriving equations for quantitation and generalization (5, 6).

METHODS

The milk proteins were separated from skim milk by acid precipitation, washed, solubilized for counting in a liquid scintillation counter, or were hydrolyzed for amino acid analyses. Casein was precipitated from skim milk with an acetate-acetic acid buffer at pH 4.45.

Barium sulfide-^{35}S was given orally as a source of sulfur for ruminal methionine synthesis in cows adjusted to controlled levels of constant feed intake. The 24-hour turnover of methionine was determined by adding to the daily rations increments of fish meal as a methionine pool diluent in the rumen. It was thereby possible to estimate the size of the methionine pool available for use in milk synthesis. New methionine available was resolved into synthesized methionine and feed methionine by regression procedures (1).

Four experiments were carried out to evaluate the effects of varying planes of nutrition.

Diets. The basal ration consisted of chopped alfalfa which had been either

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heat-dried or wilted and ensiled. The alfalfa supplied most of the dietary nitrogen.

Plane of nutrition was varied in 3 ways: 1) by selecting different levels of alfalfa intake; 2) by adding either corn meal or a semi-purified concentrate containing approximately 3.5 kcal of digestible energy per g; and 3) by varying the proportions of alfalfa and concentrate in the rations of cows eating ad libitum.

In the first replicate, experiments 1 and 2, different proportions of heat-dried alfalfa hay and ground corn were used. The rations contained 50, 60, 75 and 100% alfalfa by weight. Total feed eaten was set in these experiments as the maximal voluntary feed intake of each cow. In the second replicate, experiments 3 and 4, ensiled alfalfa was used. The level of alfalfa of each cow was adjusted to the minimal level observed among the three experimental cows so that the diets of each were essentially isonitrogenous. In experiment 3 the diets of 2 cows were enriched with ground shelled corn. Similarly in experiment 4, a semi-purified concentrate was added to the ration of 2 cows. The concentrate contained 29% cornstarch, 59% flaked soybean hulls, 2% dicalcium phosphate, 2% salt plus a trace mineral mixture [3] and 454 IU of vitamin A/454 g of feed.

Five additional cows, experiments 5 and 6, were studied to determine differences which are associated with source of nitrogen. The specific aim was to obtain data with a diet containing a high percentage of soluble nitrogen to compare with the results obtained for the heat-dried alfalfa in experiments 1 and 2 and ensiled alfalfa in experiment 3 and 4.

The test for determining solubility of nitrogen was solubility in 5% trichloroacetic acid. TCA-soluble nitrogen was 12.8% for dried alfalfa, 38% to 46% for ensiled alfalfa, and 77% for the corn silage-urea concentrate diet used in experiments 5 and 6.

In experiment 5, two cows were fed corn silage as the basal diet in which were mixed 450 g of urea and 4 kg of the semi-purified concentrate described above for experiment 4. In experiment 6, the diet of the 3 cows was similar to that of experiment 5 but was further enriched with ensiled ear corn.

All cows were allowed 21 days or more of preliminary feeding to adjust to the experimental rations.

RESULTS AND DISCUSSION

Plane of nutrition. Results from experiments 1 to 4 are presented in table 1, arranged according to the experimental design. Results from one cow in each of the 2 replicates were ajudged meaningless and excluded because the cows in question failed to eat at uniform rates, a condition necessary to maintain a steady state.

The distribution of the nutrient pool of microbially synthesized methionine and feed methionine that was not degraded in the rumen is related to feed intake, alfalfa nitrogen and nitrogen absorbability (table 1). Also shown is the methionine content of the feed and the methionine turnover in grams per day. Methionine synthesis varied among 10 cows from 10.7 to 24.1 g/day. The deviations in daily rates of synthesis became physiologically meaningful when it was found that step-by-step insertion of three fixed experimental variables (total dry matter intake, amount of alfalfa nitrogen, and type of alfalfa) and the observed variable, nitrogen absorbability, into a multiple correlation analysis accounted for most of the variation. The multiple correlation coefficient was 0.97. This statistical outcome permitted summary of the data in the following multiple regression equation:

$$M \text{ in grams} = 18 + 1.5\ (I - 9.5) + 0.067\ (A - 239) + 10.7\ (N^{1/2} - 9.6) + 0.8\ T \pm 1.4,$$

where M is the methionine synthesized, I is the total dry matter intake, A is alfalfa nitrogen, N is the absorbability of total nitrogen, T equals + 1 when alfalfa hay is fed and − 1 when alfalfa silage is fed and $\pm$ 1.4 is the deviation from regression.

It is concluded that methionine synthesis increased at the rate of 1.5 g/kg of feed consumed and 6.7 g/100 g of alfalfa nitrogen eaten.

A marked decrease in methionine synthesis was associated with reduced nitrogen absorption (table 1). The statistical

[3] Trace minerals were added to the concentrate at the following levels: (in ppm) potassium iodide, 0.3; copper oxide, 3.75; ferrous carbonate, 22.5; zinc oxide, 45; and manganese sulfate, 60.

result leads to the conclusion that this diminution in ruminal synthesis is a function of the square root of the nitrogen absorbability. The physiological meaning of this association is not discernible from the data obtained but is under investigation.

The type of alfalfa, dried or ensiled, produced differences (1.6 g of methionine/day) comparable to the residual error and therefore were not statistically significant.

The specific effect of adding the starch-cellulose concentrate to a constant intake of alfalfa in experiment 4 is shown in table 2.

Additional computations resulted in estimating a net increase of 23% in alimentary methionine. However, considerable feed methionine was degraded in the rumen. Degraded methionine was 44% or higher for dried alfalfa and 53% or higher for ensiled alfalfa. The degradation rates are in the general range of estimates obtained by other investigators (9). At the sites of absorption 57% of the absorbed methionine apparently originated from digestion of rumen microorganisms.

Sources of nitrogen. The distribution in the nutrient pool of microbially synthesized methionine and feed methionine that was not degraded in the rumen are shown in table 3. Feed intake, nitrogen intake, and other pertinent experimental variables are included also. The enriched diet, experiment 6, effected a greater synthesis of methionine which was associated with a higher percentage of grain-concentrate in that ration than was used in experiment

TABLE 1

Distribution in the nutrient pool of microbially synthesized and feed methionine not degraded in the rumen and its relationship to feed intake, alfalfa nitrogen, nitrogen absorbability in lactating cows

	Dried alfalfa			Ensiled alfalfa		
Experiment no.	1–2	2	1–2	3–4	4	3–4
No. cows	2	1	2	2	1	2
No. of trials	6	3	6	6	3	6
Feed intake, kg/day	8.84	10.56	10.01	7.38	9.60	10.95
Alfalfa intake, kg/day	8.84	7.81	5.45	7.38	7.75	7.25
Grain supplement intake, kg/day [1]	0.00	2.75	4.56	0.00	1.85	3.70
Alfalfa nitrogen intake, g/day	292	304	187	222	241	222
Daily nitrogen intake, g/day	292	338	269	222	261	260
Nitrogen digestibility, % [2]	78.1	72.5	67.6	74.9	73.2	67.5
Absorbability of nitrogen, % [3]	95.5	89.9	88.7	93.5	93.9	91.0
Feed methionine, g	26.5	33.1	26.6	20.2	24.5	26.8
Nutrient pool, g	50.0	68.5	30.6	39.3	47.3	43.5
Methionine turnover, g/day	41.2	43.7	24.4	23.6	35.9	27.2
Feed methionine undegraded, g/day	19.0	19.6	10.4	9.3	16.5	10.6
Synthesized methionine, g/day	22.2	24.1	14.0	14.3	19.4	16.6
Standard error, g	±3.8	±2.2	±2.9	±2.2	±1.5	±1.0
Synthesized methionine adjusted, g/day [4]	17.8	19.2	19.5	17.8	18.0	16.3

[1] Contained corn meal or cornstarch, flaked soybean hulls and minerals.
[2] Apparent, nitrogen intake minus fecal nitrogen divided by the nitrogen intake.
[3] Nitrogen intake minus fecal nitrogen minus endogenous fecal nitrogen divided by nitrogen intake. Fecal endogenous nitrogen estimated by method of Titus (8) as 5.6 mg/g of fecal dry matter.
[4] Synthesized methionine adjusted by a multiple covariance method for difference in feed intake, alfalfa nitrogen and nitrogen absorbability. The multiple regression equation, in its physiologically meaningful form.
M in grams $= 18.0 + 1.5\,(I - 9.5) + 0.067\,(A - 239) + 10.7\,(N^{1/2} - 9.6)$.
where M is methionine synthesized, I is total feed intake; A is alfalfa nitrogen; and N is the absorbability of total nitrogen.

TABLE 2

Increase in methionine synthesis with the addition of starchy concentrates to a constant intake of alfalfa [1]

Concentrate added, kg/day	—	1.85	3.60
Alfalfa nitrogen, g/day	241	241	241
Methionine synthesized, g/day	14.4	19.4	22.5

[1] Three trials per test.

5. Apparent ruminal degradation of feed methionine was less, 43% with the higher concentrate diet than with the lower concentrate diet used in experiment 5 in which 67% was degraded.

Results of the comparative study on sources of nitrogen are summarized in table 4. Mean values for daily methionine turnover, "feed methionine undegraded" and synthesized methionine are arranged according to basal rations, nitrogen source and TCA-soluble nitrogen. The decreases in these values are obviously inversely associated with percentage of TCA-soluble nitrogen in the ration. Conversely, assuming that all of the TCA-soluble nitrogen was nonprotein nitrogen, the residual protein nitrogen was found to be positively related to methionine synthesis. After setting other variables at their mean value, this relationship is represented by the following linear regression:

$$M = 1.35 + 0.4\,P,$$

where M is grams of methionine synthesized per kilogram of dry matter intake and P equals kilograms of intact protein consumed daily.

TABLE 3

Distribution of methionine in the nutrient pool of cows fed urea-supplemented corn silage diets, and its relationship to certain dietary components

	Corn silage	Enriched corn silage
Experiment no.	5	6
No. of cows	2	3
No. of trials	6	9
Dry matter intake, kg/day	10.9	8.9
Corn silage, intake, kg/day	8.0	6.2
Grain concentrate in diet, %	62.5	74.0
Urea nitrogen, g/day	168	176
Total nitrogen, g/day	302	291
Nitrogen digestibility, %	78.9	75.5
Absorbability of nitrogen, %	91.5	86.6
Nitrogen balance, g/day	17.2	23.4
Feed methionine, g/day	16.0	14.4
Nutrient pool, g	28.6	44.1
Methionine turnover, g/day	17.0	26.0
Feed methionine undegraded, g/day	5.3	9.4
Synthesized methionine, g/day	11.7	16.6
Standard error, g	±3.6	±2.4

TABLE 4

Distribution of methionine in the nutrient pool and its relationship to feed intake, TCA-soluble nitrogen and absorbability of nitrogen [1]

Basal diet	Dried alfalfa	Ensiled alfalfa	Corn silage
Experiment no.	1–2	3–4	5–6
Dry matter intake, kg/day	9.7	9.3	9.7
Alfalfa nitrogen, g/day	252	226	0
Urea nitrogen, g/day	0	0	173
TCA-soluble nitrogen, %	10.4	38.5	76.5
Total nitrogen, g/day	291	245	295
Nitrogen digestibility, %	72.8	71.5	76.9
Absorbability of nitrogen, %	91.4	92.6	88.6
Feed methionine, g/day	27.9	23.7	15.1
Nutrient pool, g	45.6	42.5	37.9
Methionine turnover, g/day	34.9	27.5	22.2
Feed methionine undegraded, g/day	15.6	11.1	7.3
Synthesized methionine, g/day	19.3	16.4	14.7
Standard error, g	±2.3	±1.3	±2.5

[1] Fifteen trials per ration.

It is concluded that maximal synthesis of methionine by cows fed a particular ration is a function of the quantity of intact protein consumed. Since rumen microorganisms are not known to have obligatory requirements for organically bound nitrogen (10), it is suspected that the enhanced synthesis that was positively correlated with intact protein is referable to an optimal rate of production of ammoniacal nitrogen, that is, microorganisms degraded protein at about the same rate that they used nitrogen for growth.

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Prediction of Metabolizable Energy in Preadolescent Children [1]

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ABSTRACT Metabolizable energy (ME) of preadolescent girls can be predicted accurately from gross energy (GE) intake and dietary nitrogen (DN). Three formulas, based on dietary composition, have been developed using data obtained from 58 subjects on controlled metabolic experiments containing various amounts and quality of protein. The following prediction formula covers the most extensive range in dietary composition of these experiments:
ME = 0.9746 (GE) − 4.28 (DN) − 57.24.

Estimation of metabolizable energy by determination of caloric intake and excretion by bomb calorimetry is a tedious and time-consuming process requiring specialized facilities and procedures. Hence, more convenient but less precise methods have often been used to eliminate some of these disadvantages. Such methods include calculation of dietary intake by the use of food composition tables with a standard allowance of 10% for energy lost in excreta (1). A more accurate method used involves analysis of the fat, protein, and carbohydrate content of the diet and calculations of metabolizable energy using Atwater's physiological fuel values. Values of 4 kcal/g of protein and carbohydrate and 9 kcal/g of fat allow a correction for energy loss of ingested foodstuffs in excreta. The primary limitation to this method is the tediousness of the chemical determinations.

A method of estimating metabolizable energy in humans based on caloric and nitrogen intakes has been devised by the Medical Nutrition Laboratory, U. S. Army (2, 3). Since this formula is limited to adults, the authors have developed similar formulas to predict metabolizable energy of normal preadolescent girls under various dietary conditions (4, 5). The development and evaluation are presented in this paper.

Metabolizable energy (ME) is defined as the amount of energy available for use after the gross food energy has been corrected for energy lost in urine and feces. Analyzed or determined metabolizable energy refers to that calculated from values obtained by bomb calorimetry of food, urine, and feces.

DESCRIPTION OF STUDIES

In the fall of 1954, and the summers of 1956, 1957, 1958 and 1962, groups of 7- to 9-year-old girls participated in controlled dietary experiments. The general plan and organization, selection and management of the subjects, dietary regimens, physical and biochemical measurements and analytical procedures have been described in detail (4, 5). Table 1 presents an outline of the experiments and gives the mean caloric and nitrogen intakes for each diet used. The experiments varied in length from six 6-day periods (36 days) in 1962 to sixteen 4-day periods (64 days) in 1954. A different menu was used on each day within each balance period for each diet; this meant a total of 48 menus, not including pre-experimental periods for which the data were eliminated.

Dietary descriptions and design. The daily energy balance data were collected

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[1] This study was part of the Southern Regional Research Project, S-28. Requirements and Utilization of Selected Nutrients by Preadolescent Children, supported in part by funds appropriated to the USDA under the Research and Marketing Act of 1946 and the Hatch Act, as amended. The Human Nutrition Division of the Agricultural Research Service was a cooperator in this project. A series of papers has been published under the title, "Metabolic Patterns in Preadolescent Children."

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TABLE 1

Description of metabolic studies

Year	Diet	No. of subjects	Mean wt [1]	Experimental plan	Energy intake [1]	N intake [1]	Dietary plant protein
			kg		*kcal/day*	*g/day*	*%*
1954	1	11	30	60 g protein	1982	10.50	32
1956	2	3	24	Light wt, 2 g protein/kg	1977	7.70	30
	3	3	27	Light wt, 3 g protein/kg	1966	11.64	22
	4	3	31	Heavy wt, 2 g protein/kg	2374	9.39	28
	5	3	33	Heavy wt, 3 g protein/kg	2415	14.12	19
1957	6	6	28	1.25 g protein/kg	2412	6.16	34
	7	4	36	1.25 g protein/kg	2244	7.35	29
1958	8	13	28	0.8 g protein/kg (periods 1–5)	2176	3.54	49
	8′	13 [2]	29	0.6 g protein/kg (periods 6–8)	2244	2.29	61
1962	9 and 10	6	27	0.8 g protein/kg	2200	3.54	100
	11 and 12	6	29	1.4 g protein/kg	2320	6.34	100

[1] Mean values presented in this table were obtained from individual observations for subjects in periods for which complete data were available. In some cases complete data were not available for a subject, therefore the means given here may differ slightly from values published previously.
[2] These were the same subjects that were receiving diet 8.

over a wide range of protein intake from a low of 18 g to a high of 88 g. The percentage ratio of animal protein to vegetable protein ranged from 75:25 to 0:100. The daily energy intakes ranged from 1966 to 2415 kcal; these included values below and above the National Research Council's recommended 2000 kcal and 60 g of protein daily for the 27-kg 8- to 9-year-old reference child (6, 7). These were the recommendations in use when the studies were initiated.

PROCEDURES

Energy values for food were determined by bomb calorimetry under various experimental conditions (4, 5). Energy determinations were made in an Emerson bomb calorimeter and nitrogen determinations were made by the Kjeldahl technique.

Data from the above experiments were used to develop three regression formulas for predicting metabolizable energy. The first was based on data obtained from subjects fed mixed protein diets (306 observations). A second formula for predicting ME with vegetable protein diets was developed (72 observations). The third formula was based on all experimental data (378 observations) in which the plant protein content of the diet ranged from 19 to 100% of the total protein intake (table 1). The model for the formulas is $\hat{y} = a + b_1x_1 + b_2x_2$, where a is the y intercept, b_1 and b_2 are the appropriate partial regression coefficients, x_1 is gross energy intake (GE) in kilocalories per day and x_2 is dietary nitrogen (DN) in grams per day (8). Formulas were derived in which both a correction and no correction were made in ME for nitrogen balance; for these data, the correction for nitrogen balance was found to be unnecessary (2).

Each formula was applied to the data from which it was derived to calculate the predicted ME. The variance due to deviations between predicted and observed values, expressed as a fraction of the total variance in y, is estimated by R^2. In order to evaluate the fit of the formulas to the data, the difference between the predicted and observed ME was calculated and expressed as percentage error; that is, $\%\ \text{error} = \frac{\hat{y} - y}{y} \times 100$. A positive percentage error indicates that the formula overpredicted ME whereas a negative value indicates that the formula underpredicted ME.

To test this method of predicting ME, the formula derived from all the data was applied to selected subjects from Macy's studies (9), ranging in age from 6.5 to 10.5 years, who had nitrogen and caloric intakes within the range of this study. The difference between the analyzed and predicted values are shown for these data (table 2). Comparisons between deter-

TABLE 2

Difference between determined and predicted metabolizable energy for experimental subjects and selected Macy (9) subjects

% error [1]	% of observations			
	Formula 1 [2]	Formula 2 [3]	Formula 3 [4]	Macy data [5]
−3		3	2	1
−2	8	11	25	18
−1	46	40	16	23
1	38	31	36	35
2	6	10	11	17
3	1	4	3	5
4	0.3		4	1
5		1	1	
6			1	
7	0.3		0.5	

[1] $\%\ \text{error} = \frac{\text{predicted ME} - \text{determined ME}}{\text{determined ME}} \times 100.$
[2] Diets contained 19–61% vegetable protein.
[3] Diets contained 100% vegetable protein.
[4] Diets contained 19–100% vegetable protein.
[5] This includes children of both sexes (9).

mined ME and ME calculated from both the physiological fuel values and the prediction formula were made to evaluate the effectiveness of the prediction formula.

RESULTS

The following prediction formulas were derived for the three dietary categories. Table 2 shows the range of differences between the determined and predicted ME and the percentage of cases in each category.

1. Mixed protein diet with g N/day ranging from 2 to 14 and kcals/day ranging from 1948 to 2608. The formula obtained for predicting metabolizable energy for subjects fed mixed protein diets (1954, 1956, 1957 and 1958) is:

$$ME = 1.005\ (GE) - 5.940\ (DN) - 98.417 \quad (1)$$

In this equation 99.4% of the variation in ME was accounted for by the two dietary components ($R^2 = 0.994$). For 98% of the data, the formula predicted ME within ± 2% of the analyzed value.

2. Vegetable protein diet. The formula for predicting ME for subjects on vegetable protein diets (1962) is:

$$ME = 0.9181\ (GE) - 16.74\ (DN) + 81.82 \quad (2)$$

In this equation, GE and DN accounted for 97.6% of the variation in ME ($R^2 = 0.976$). Ninety-two per cent of the observations fell within ± 2% error, whereas 99% fell within ± 3% error.

3. Diets containing 19 to 100% vegetable N (1954–1962). Metabolizable energy can be predicted as follows:

$$ME = 0.9746\ (GE) - 4.2782\ (DN) - 57.2389 \quad (3)$$

In this equation 97.6% of the variation in ME was accounted for by the independent variables ($R^2 = 0.976$). Eighty-eight per cent of the predicted values are within ± 2% of the observed ME, whereas 93% are within ± 3%.

The results of applying this prediction formula to selected Macy data are shown in table 2. The formula was effective in predicting 99% of the data within ± 3% of the observed ME; 93% were within ± 2% of the observed value.

DISCUSSION

Predicting ME from caloric and protein intakes. These results indicate that metabolizable energy of preadolescent girls can be adequately predicted from gross caloric intake and nitrogen intake with a maximum of 4% of the variation due to other factors. The accuracy of predicting ME decreased slightly with the inclusion on the data in which vegetable protein was 100% of the total protein intake. This indicated that the results obtained from feeding vegetable protein diets were not strictly compatible with those in which mixed protein was fed. Fecal loss for subjects receiving the vegetable protein diets was

greater than for subjects receiving the mixed protein diets. When using equation 1 to predict ME, 99% of the data fell within ± 3% error (table 2); if equation 3 was used 93% of the data fell within ± 3% error. When using equation 2 to predict ME on a vegetable protein diet, 99% of the values were within ± 3% of the determined values.

Application to data from Macy's studies. In evaluating the effectiveness of the prediction formula on data obtained by Macy on subjects within the 7- to 10-year age group (9), formula 3 was found to be the most effective predictor. Ninety-nine per cent of the observations were predicted within ± 3% error (table 2). Although there was more variation in formula 3 when applied to the data from which it was derived, it appeared to be a better general prediction formula. This was probably because of the wider range of dietary intakes and composition on which this formula was based.

The predicted ME of these subjects was compared with the observed ME and with that calculated using the physiological fuel values (fig. 1). In the age range of 6.5 to 8.5 years, the predicted ME was closer to the determined ME than the values obtained using the Atwater factors. From ages 9.0 to 10.5, the two indirect methods of calculating ME were about the same in relation to the determined ME.

Limitations in using the formulas. Use of the formulas to predict ME would be limited to situations similar to those under which they were derived; that is, for healthy girls, 7 to 10 years of age, ingesting diets containing 1966 to 2415 kcal/day and 2 to 14 g N/day. The application of one of the formulas to data obtained by Macy (9) from children of both sexes showed that it could be used for children ingesting normal diets containing kilocalories and protein within the experimental ranges. None of the formulas have been applied to data obtained under any extreme dietary conditions. Since less than 4% of the ME was found to be influenced by factors other than dietary nitrogen and caloric intake, it is theoretically possible that the formula based on the vegetable protein diets would be applicable to situations in countries where diets are composed only of plant products.

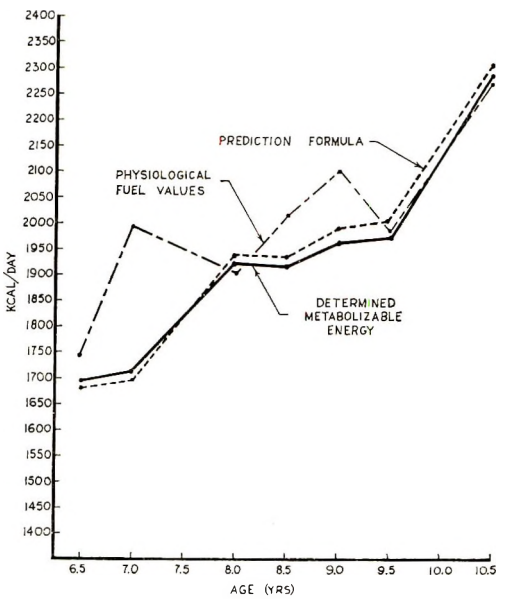

Fig. 1 Comparison of the values determined by bomb calorimetry with the calculated values by the prediction formula and the physiological fuel values for selected Macy data.

The 3 formulas can be used to accurately predict metabolizable energy for preadolescent girls with minimal time spent for chemical analyses of gross energy intake and dietary nitrogen. The ME can be predicted within an average of ± 2% error and with a maximum of ± 4% error. The precision to which the formulas predict is within ± 40–80 kcal on a daily intake of 2000 kcal. In an average day's diet, this caloric variation amounts to about 10 g (2 teaspoons) of butter, 23 g (1 slice) of bread or 12 g (1 tablespoon) of sugar. In view of the sources of error which can arise from complete energy determinations by bomb calorimetry, this is a minute amount. Although these prediction formulas will not replace direct determination of energy balance by bomb calorimetry, they could be used in situations where it is undesirable to do the extensive determinations necessary to calculate energy balance.

ACKNOWLEDGMENTS

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Water Fluoridation: Effect on bone fragility and skeletal calcium content in the rat [1]

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ABSTRACT The calcium content of the axial skeleton and the forelimb is a linear function of body weight and is increased in rats when they drink water containing 20 ppm of fluorine in the form of sodium fluoride, but not in the hindlimb nor when they drink water containing 2 or 5 ppm of fluorine. Breaking force of the humerus and femoral shaft is a linear function of body weight and is unaffected by water fluoridation up to a concentration of 20 ppm.

Fluoridation of water has long been known to cause qualitative changes in the teeth of animals and humans (1). In recent years reports have appeared of the therapeutic use of "fluoride" in Paget's disease of bone (2) and in osteoporosis (3). The latter is based upon the observation that osteosclerosis occurs in chronic fluorosis. It has been postulated that fluoridation of water might possibly benefit not only dental health but also might mitigate against the osteoporosis that commonly affects aging women (4).

It is the purpose of the present paper to report quantitative and qualitative changes that occur in the skeleton of the rat with age and varying intake of "fluoride" from weaning to maturity.

MATERIALS AND METHODS

Eighty-nine male rats of the Charles River CD strain, 25 days old, averaging 67 g, were divided into 4 groups so that the group averages were within one gram of each other. They were fed a commercial purified diet.[2] This diet contained no added "fluoride," manganese, copper, zinc or iodine. Our analysis of 6 samples of this diet showed an average calcium concentration of 0.43%. The diet was submitted to neutron activation analysis and fluorine content was not detectable by this method, indicating a concentration of less than 2 ppm. X-ray fluorescence spectroscopy revealed the presence of manganese and copper but not of zinc or iodine. The animals were housed in galvanized wire cages and fed ad libitum the diet, which was pressed into pellets. They were given distilled water to which was added sodium fluoride so as to give a concentration of 2, 5 and 20 ppm, respectively, of fluorine in groups 2, 3 and 4. No "fluoride" was added to the water of group 1 which acted as control.

The animals were killed five at a time from each of the 4 groups at predetermined weights. The average weight of the animals killed did not differ significantly from the average weight of the remainder of the group and hence did not change the average weight of the groups. The experiment ended with the killing of the last of the animals on day 98 when these animals were 123 days old. When the experiment started on the day 25, the average weight of each of the 4 groups was 67.2 g, 67.6 g, 67.0 g, and 67.4 g, respectively. At the end of the experiments when the animals were 123 days old, their average weights were 408 g, 424 g, and 405 g, respectively.

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[2] Percentage composition of diet: vitamin-free casein, 18; sucrose, 68; vegetable oil, 10; and salt mixture no. 2, USP 13, 4 (Vitamin B Complex Test Diet-Complete, Nutritional Biochemicals Corporation, Cleveland). It was supplemented with one kilogram of vitamin mixture per 45.5 kg of diet. The vitamin mixture contained: (in grams) vitamin A conc (200,000 U/g), 4.5; vitamin D conc (400,000 U/g), 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; Ca pantothenate, 3.0; and (in milligrams) biotin, 20.0; folic acid, 90.0; and vitamin B_{12}, 1.35 (Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation).

The fore and hindlimbs were disarticulated at the shoulder and hip, respectively. The dismembered carcasses were eviscerated. Soft tissues were dissected from the femur and humerus from one side of the animal for measurement of breaking force. The other femur, the other upper limb and the lower limb excluding the femur were ashed separately as was the remainder of the dismembered carcass at 525° for from 24 to 48 hours, dissolved in 2 N hydrochloric acid and made up to volume in a volumetric flask. Calcium was determined by a semi-automatic EDTA method (5). Breaking force measurements were carried out on the entire humerus and on the distal half of the femoral shaft. The part of the femoral shaft used was the more-or-less cylindrical portion which lies between the end of the ridge of the third trochanter and the condyles at the distal end of the bone. The ends of each bone sample were imbedded by means of epoxy cement into plastic discs (approximately 1.3 cm in diameter and 0.6 cm in thickness). A shallow well to receive the bone was drilled into one face of each disc. A compressive force was applied to the sample through the discs to provide an even distribution of force across the ends of the bone. The mounted femur samples were dried in an oven maintained at 60° for a period of 48 hours (72 hours for the larger sample). The dried samples were then placed in a dessicator until testing. The humerus samples were soaked in physiological saline which was degassed in a vacuum chamber for 1.5 hours. The mounted samples were tested to failure under compression in a Riehle material testing machine [3] calibrated to a precision of 1%.

Since the calcium content of individual long bones (6) as well as the total rat carcass (7) are linear functions of the body weight of the animals, by plotting the calcium content of various parts of the skeleton as a function of body weight and calculating the regressions so formed, it is possible by a comparison of these regressions to show differences in mineral content in part or all of the skeletons of animals under varying circumstances. We have also found that the breaking force of the femur and humerus, whether measured dry or wet, is a linear function of body weight in the rat so that the same statistical methods can be used to compare bone fragility in animals having a varying "fluoride" intake (8).

Regressions were calculated by the method of least squares. These procedures and the subsequent analyses were performed as described by Snedecor (9).

RESULTS

The calcium content of the eviscerated dismembered carcasses (axial skeleton) as well as of the limb bones was compared in the 4 groups of animals. Examples of these regressions are graphed in figures 1 and 2. The regression equations are given in tables 1, 2, 3 and 4. The analysis reflects the design of the study in that each group is compared with the basic line or control group. The axial skeleton of rats drinking water containing 20 ppm of "fluoride" contained significantly more cal-

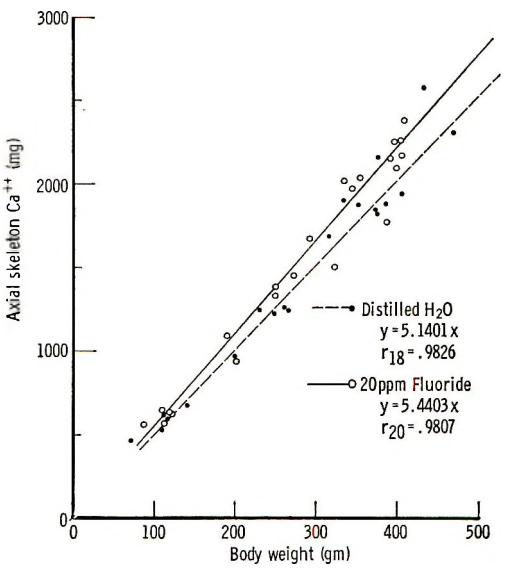

Fig. 1 Calcium of axial skeleton is plotted against body weight for groups 1 and 4. The regressions were calculated by the method of least squares. Since the intercepts did not differ significantly from zero, the lines were drawn through zero and the simplified equation $Y = kx$ given. The regressions were compared by analysis of covariance and the intercepts were found to differ significantly from each other ($0.05 > P > 0.01$) but the scatter and slopes did not.

[3] Model PS-10 Universal Screw Power testing machine (0–5000 kg) Riehle Testing Machines, a division of Ametek, Inc., East Moline, Illinois.

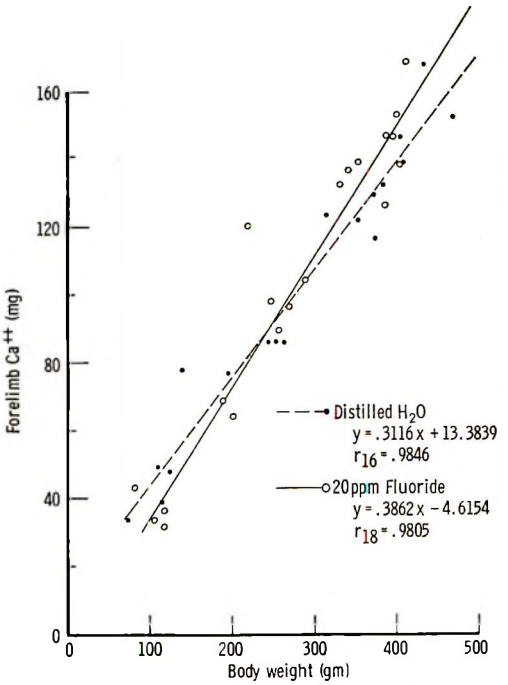

Fig. 2 Forelimb calcium is plotted against body weight for groups 1 and 4. The regressions were calculated by the method of least squares and compared by analysis of covariance. There was a significant slope difference ($0.01 > P > 0.001$).

TABLE 1

Regression of calcium content of axial skeleton (Y) vs. body weight (X) [1]

Group no.	Water content	$Y - \bar{Y} = k\,(X - \bar{X})$
1	distilled water	$Y - 1432 = 5.140\,(X - 279.3)$
2	2 ppm F^-	$Y - 1523 = 5.465\,(X - 278.4)$
3	5 ppm F^-	$Y - 1475 = 5.275\,(X - 281.7)$
4	20 ppm F^-	$Y - 1524 = 5.440\,(X - 279.5)$

[1] Regression 1 was compared with regressions 2, 3 and 4, respectively. Regression 4 had a significantly higher intercept than regression 1. There was a non-significant difference between either regressions 1 and 2, or 1 and 3.

TABLE 2

Regressions of calcium content of forelimb (Y) vs. body weight (X) [1]

Group no.	Water content	$Y - \bar{Y} = k\,(X - \bar{X})$
1	distilled water	$Y - 100.9 = 0.3116\,(X - 280.7)$
2	2 ppm F^-	$Y - 109.0 = 0.3566\,(X - 284.5)$
3	5 ppm F^-	$Y - 101.0 = 0.3436\,(X - 281.3)$
4	20 ppm F^-	$Y - 104.3 = 0.3862\,(X - 282.0)$

[1] Regression 1 was compared with regressions 2, 3 and 4, respectively. Regression 4 had a significantly higher slope (k) than regression 1. There was a non-significant difference between either regressions 1 and 2, or 1 and 3.

TABLE 3

Regressions of calcium content of femur (Y) vs. body weight (X) [1]

Group no.	Water content	$Y - \bar{Y} = k\,(X - \bar{X})$
1	distilled water	$Y - 92.9 = 0.3760\,(X - 285.4)$
2	2 ppm F^-	$Y - 93.0 = 0.3969\,(X - 279.6)$
3	5 ppm F^-	$Y - 80.9 = 0.3843\,(X - 255.5)$
4	20 ppm F^-	$Y - 91.8 = 0.3790\,(X - 275.8)$

[1] There were nonsignificant differences between the regression for group 1 and regressions for any of the other groups.

TABLE 4

Regressions of calcium content of hind leg without the femur (Y) vs. body weight (X) [1]

Group no.	Water content	$Y - \bar{Y} = k\,(X - \bar{X})$
1	distilled water	$Y - 166.2 = 0.5954\,(X - 278.7)$
2	2 ppm F^-	$Y - 168.9 = 0.6016\,(X - 279.7)$
3	5 ppm F^-	$Y - 140.0 = 0.5868\,(X - 238.2)$
4	20 ppm F^-	$Y - 163.3 = 0.5779\,(X - 276.9)$

[1] There were nonsignificant differences between the regression for group 1 and regressions for any of the other groups.

cium at any given body weight than did the controls ($0.05 > P > 0.01$). There was no significant difference between the controls and either those animals drinking water containing 2 ppm or 5 ppm of "fluoride." Similarly, the calcium content of the

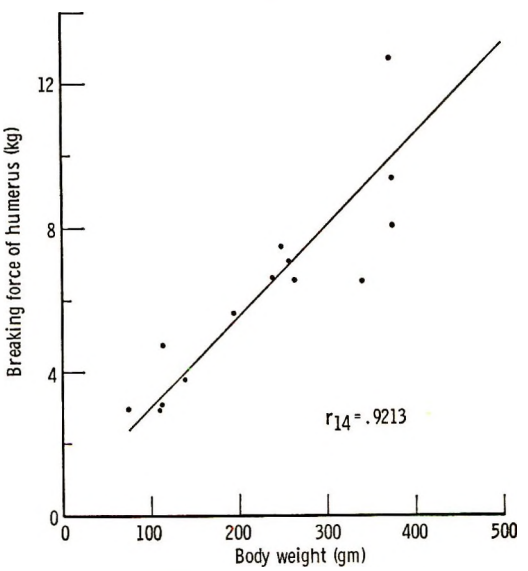

Fig. 3 Breaking force of humerus of group 1 is plotted against body weight of animal (distilled water). The line best fitting this regression was calculated by the method of least squares.

entire disarticulated forelimbs increased more rapidly as body weight increased in the rats drinking water containing 20 ppm than in the controls ($0.01 > P > 0.001$). Figure 2 illustrates these regressions. Under a body weight of about 250 g, the forelimbs of rats drinking distilled water contain more calcium than the forelimbs of rats drinking water containing 20 ppm of "fluoride"; but above this body weight the reverse is true; the fluoridated animals had significantly more calcium in the forelimbs. There was no significant difference between the calcium content of the femurs of the controls and the other 3 groups nor between the calcium content of the remaining bones of the leg in the controls compared with the other 3 groups. Figures 3 and 4 show how the breaking force of the humerus and of the femur, respectively, are linear functions of body weight. The regression equations for breaking force of bones versus body weight are listed in tables 5 and 6. There were nonsignificant differences in breaking force of the humeri and the femurs at any given body weight between the animals drinking distilled water and the 3 groups drinking fluoridated water.

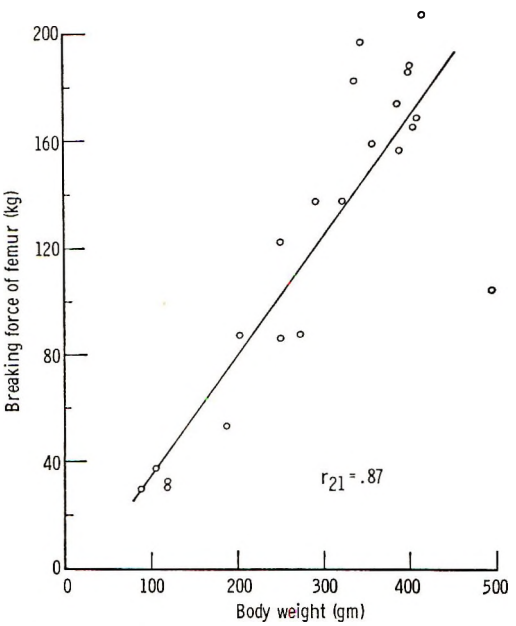

Fig. 4 Breaking force of femoral shaft in group 4 is plotted against body weight of animal (20 ppm fluoride). The line best fiitting this regression was calculated by the method of least squares.

DISCUSSION

Fluorine is a bone-seeking element. In the rat, the concentration found in the skeleton depends partly on the amount ingested but also on the rate of growth of the animal: rapidly growing animals retain more "fluoride" than slowly growing animals (10). Furthermore, the concentration of "fluoride" varies in different parts of the skeleton so that the vertebrae have a

TABLE 5

Regressions of breaking force of femoral shaft (Y) vs. body weight (X) [1]

Group no.	Water content	$Y - \overline{Y} = k(X - \overline{X})$
1	distilled water	$Y - 98.95 = 0.4257\ (X - 279.6)$
2	2 ppm F^-	$Y - 132.05 = 0.5530\ (X - 283.4)$
3	5 ppm F^-	$Y - 112.49 = 0.4709\ (X - 273.4)$
4	20 ppm F^-	$Y - 119.48 = 0.4462\ (X - 288.9)$

[1] Regression 1 was compared with regressions 2, 3 and 4, respectively. Nonsignificant differences were found.

TABLE 6

Regressions of breaking force of humerus (Y) vs. body weight (X)

Group no.	Water content	$Y - \overline{Y} = k(X - \overline{X})$
1	distilled water	$Y - 7.181 = 0.0251\ (X - 266.7)$
2	2 ppm F^-	$Y - 7.515 = 0.0296\ (X - 253.7)$
3	5 ppm F^-	$Y - 7.087 = 0.0253\ (X - 262.3)$
4	20 ppm F^-	$Y - 7.831 = 0.0291\ (X - 267.6)$

[1] Regression 1 was compared with regressions 2, 3 and 4, respectively. Nonsignificant differences were found.

higher concentration than the femora (11). There is some evidence to suggest that the effect of "fluoride" on bone is dependent on bone fluoride attaining a critical level (12, 13). Therefore, it is not unexpected to find an increase in the calcium content of the axial skelton but not in the bones of the hindlimb which are largely cortical in type. Rich and Ivanovich (14) performed densitometric studies on a single patient suffering from osteoporosis who was given "fluoride" over a period of 122 weeks. They found that there was an increase in bone density following treatment, most marked in the lower end of the ulna but less in the oscalcis and that there were almost no changes in the phalanges. This report in a human is consistent with the observations reported here in rats. It is of interest that when young rats drink water containing 20 ppm of "fluoride," the disarticulated forelimbs contain less calcium than the control animals, but in older animals the forelimb calcium in the fluoridated animals becomes progessively greater than the controls. Morphological studies would be needed to elucidate this point further; however, one explanation might be that osteomalacia-like changes are induced in the bones of young animals by "fluoride." Yet, if this were the true explanation, similar results would be expected elsewhere in the skeleton and these were not found. Another explanation could be delay in maturation of ossification centers in young animals, but once ossification had started, more dense bone was laid down in the forelimbs of animals consuming water containing 20 ppm of "fluoride" than in the controls. Finally, the apparent decrease in forelimb calcium in young animals receiving "fluoride" may be an artifact of the method used for analysis in which regression lines may misrepresent the region of one extreme.

There have been few studies documenting the effect of "fluoride" on skeletal fragility. The method reported in this paper in which breaking force of the femur or humerus is highly correlated with body weight lends itself to comparison of breaking force between groups so that even small changes in bone fragility would be detectable. It is of interest to learn that breaking force of the humerus and of the femoral shaft is not affected by "fluoride" in a concentration up to 20 ppm in the drinking water. The reason for this may be that breaking force tested in this way depends very largely on the quality and quantity of cortical bone which has not been affected at this dose level of "fluoride."

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A Comparative Study of the Nutritional and Physiological Significance of Pure Soybean Trypsin Inhibitors and of Ethanol-extracted Soybean Meals in Chicks and Rats

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ABSTRACT Several experiments were conducted with the pure soybean trypsin and α-chymotrypsin inhibitor (AA) and the crystalline Kunitz soybean trypsin inhibitor (CSBTI) to study their specific effects on growth rate of chicks and rats, on pancreatic hypertrophy and on intestinal proteolytic activity. For comparative purposes raw soybean meal (RSBM), heated soybean meal (HSBM) and the residues after ethanolic extraction of RSBM (EERSBM) were used. The addition of the inhibitors to HSBM diets of chicks and rats resulted in a slight depression of growth rate but caused a significant hypertrophy of the pancreas. However, the RSBM+HSBM diet (1:2) which possessed the same trypsin-inhibiting activity and approximately 40% of the chymotrypsin-inhibiting activity as the HSBM+AA diet, caused a considerable growth depression. The proteolytic activity in the small intestine was decreased by addition of inhibitor AA but not by CSBTI. This probably arises from the small activity of CSBTI towards chymotrypsin (8% of that of AA) and from its inactivation during peptic digestion. The comparative stability of AA was also indicated by the various ethanolic extractions of RSBM during which AA maintained its full potency, whereas CSBTI was almost completely inactivated. Addition of methionine to the different meals at two protein levels (25% and 15%) resulted in a decrease in growth impairment caused by the low protein RSBM diets. These experiments clearly indicate the minor role of soybean trypsin and chymotrypsin inhibitors in growth depression caused by RSBM diets and suggest the presence of other responsible factor(s) in RSBM.

Raw soybean meal (RSBM) causes growth inhibition and pancreatic hypertrophy when used as a source of proteins for animals (1). Various investigators have attributed these phenomena to the presence in RSBM of heat-labile trypsin inhibitors (2, 3), whereas others could not find a clear correlation between the presence of the latter in the diet and growth depression (4, 5). In an earlier report (6) we have shown that the insoluble residue of RSBM at pH 4.2, which was of high protein content and retained approximately 50% of the original trypsin-inhibiting activity of RSBM, was only slightly superior to RSBM when added to diets of chicks and rats. This controversy as to which factor or fraction in RSBM is responsible for a certain anti-nutritional or physiological effect in the animal, seems to have arisen because most of the investigations were carried out either with crude or partially purified soybean trypsin inhibitors or with Kunitz's (7) crystalline soybean trypsin inhibitor (CSBTI) but not with the pure soybean trypsin- and α-chymotrypsin-inhibitor AA (8, 9), which is stable towards peptic digestion. It was the aim of the present investigation to use the two pure and markedly different soybean trypsin inhibitors, CSBTI and inhibitor AA (7–9), as a means of elucidating the effect of these materials on pancreatic hypertrophy and on growth depression and to seek the correlation between the two. Another objective of this study was to determine the effect of certain treatments, such as extractions with ethanol under different conditions, on the removal or inactivation of these biologically active factors.

MATERIALS AND METHODS

Soybean meals and soybean trypsin inhibitors. Raw soybean meal (RSBM) was

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

Properly heated soybean meal (HSBM)[1] was a commercial product with a nitrogen content of 8.0%.

Ethanol extracted soybean meals (EERSBM) were prepared by using 60% and 80% ethanol, both at 25° and 55°. Each treatment was performed by two successive ethanolic extractions of finely milled RSBM, with constant stirring for one hour, using a 10:1 solvent-to-meal ratio for the first extraction and a 5:1 ratio for the second extraction. The residues were recovered by centrifugation at 1500 × *g* for 10 minutes, collected and dried overnight at 35°. The trypsin-inhibiting, α-chymotrypsin-inhibiting, and hemagglutinating activities of the various meals, after 2 and 18 hours of extraction, were determined by methods described later.

The consecutive supernatants of the 80% ethanolic extracts obtained from 1 kg of RSBM were combined and concentrated in vacuum to 500 ml. The concentrate was then added to 1 kg of HSBM, properly mixed and dried overnight at 35°. A preparation amounting to 1.12 kg and consisting of 1 kg HSBM plus the dry residues of an 80% ethanolic extract of 1 kg RSBM at 55° or at 25°, was thus obtained.

For the determination of the effect of ethanolic extraction of RSBM on the removal and stability of soybean trypsin inhibitors, 10 g of finely ground RSBM were extracted by 100 ml of 60% ethanol at 25° or 55° during 2 or 18 hours and then centrifuged at 1500 × *g* for 10 minutes. The recovered residue was suspended again in 1000 ml water. After 2 hours of stirring at room temperature the insoluble residue was precipitated by centrifugation at 1500 × *g* for 15 minutes and removed. The supernatants, aqueous as well as ethanolic, were assayed for trypsin- and chymotrypsin-inhibiting activities after appropriate dilutions.

The trypsin inhibitor AA was prepared according to Birk et al. (9). A 60% ethanolic extract of RSBM obtained at 55° was used for preparation of this inhibitor, since it gave a higher yield (10). Crystalline soybean trypsin inhibitor (CSBTI) was a commercial product.[2] Trypsin- and α-chymotrypsin-inhibiting activities were determined by the casein digestion method of Kunitz (7) and expressed in trypsin-inhibitor units (TIU) and in chymotrypsin-inhibitor units (ChIU), respectively (8, 10). Hemagglutinating activity was determined by the photometric method developed by Liener (11) and expressed in hemagglutinating units (HU); and proteolytic activity of the small intestine of chicks was determined by the casein digestion method of Kunitz (7) as modified by Nitsan and Alumot (12). The tryptic and chymotryptic activities in the small intestine were determined in the supernatant of homogenated chyme (12). Tryptic activity was assayed on N-benzoyl-L-arginine-ethyl ester (BAEE)[3] as substrate, using the spectrophotometric methods described by Schwert and Takenaka (13).

Determination of enzymatic activities in chick pancreas. The chicks were killed by rapid bleeding and the pancreatic tissue was immediately removed, cleaned of fat, weighed and stored at −18°. The frozen tissue was minced, homogenized in 20 vol of distilled water and centrifuged at 8000 × *g* for 10 minutes at 30°. Tryptic and chymotryptic activities were determined in the clear supernatant by the methods (13) described above, after activation of the enzyme solution via incubation of the supernatant with one volume of 0.02% purified enterokinase[4] dissolved in 0.02 M sodium phosphate buffer, pH 5.8, during 45 minutes at 37°.

Feeding trials with chicks and rats. One-day-old male White Leghorn or New Hampshire × White Leghorn chicks were fed sorghum grains for 4 or 5 days. They were then weighed individually, distributed into groups of 10 or 20 chicks in one pen for each feed treatment and housed in electrically heated battery brooders with raised wire floors. The chicks were grown with an all-vegetable diet, the main protein source being the different experimen-

[1] Prepared at Izhar Oil Industry of Israel Ltd., by heating the defatted flakes at 80° to 100° for 20 minutes, steaming for 25 minutes at 118° and drying at room temperature.
[2] Worthington Biochemical Corporation, Freehold, New Jersey.
[3] Sigma Chemical Company, St. Louis.
[4] Nutritional Biochemicals Corporation, Cleveland.

TABLE 1
Composition of experimental diet for chicks

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

[1] Alphacel, Nutritional Biochemicals Corp., Cleveland.
[2] Composition in per cent: NaCl, 94.70; MnO, 3.36; KI, 0.06; $Cu(OH)_2$, 0.14; ZnO, 0.09; $FeCO_3 \cdot 2H_2O$, 0.33; Na_2MoO_4, 0.01; and Ca stearate to make 100.
[3] Composition per 2 g: (in milligrams) thiamine·HCl, 5; riboflavin, 5; niacin, 60; Ca pantothenate, 25; pyridoxine·HCl, 5; folic acid, 4; biotin, 0.2; vitamin K, 0.5; and tocopheryl acetate, 30; and vitamin B_{12}, 20 μg; vitamin D_3, 480 IU; and vitamin A, 5000 IU.
[4] In certain diets DL-methionine was omitted.
[5] Given to supply 17, 22 or 25% protein (6.25 × N) according to nitrogen content of various meals.
[6] Composed of equal amounts of dextrose and potato starch.

tal soybean meals, with or without added trypsin inhibitors. The composition of the experimental diet is shown in table 1. Food and water were given ad libitum and the trials were terminated after 7 to 10 days.

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

As to the amounts of soybean trypsin inhibitors added to the diets, 5.5 mg of inhibitor AA were added per gram of HSBM which corresponds to the calculated inhibitor AA content in RSBM. In several experiments 16.5 mg inhibitor AA were added per gram of HSBM, yielding a diet which contained the same in vitro total trypsin-inhibiting activity as a corresponding diet with RSBM as the protein source.

CSBTI was added in amounts corresponding to those of inhibitor AA on a weight basis.

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

TABLE 2
Composition of experimental diets for rats

	%
Dextrose	33.1
Potato starch	33.1
Soybean oil	8.0
α-Cellulose [1]	1.0
Salt mixture no. 2, USP [2]	4.0
Vitamin mixture [3]	0.3
Choline chloride	0.2
DL-Methionine	0.3
Soybean protein source [4]	20.0

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[2] Nutritional Biochemicals Corporation.
[3] Compositon per 3 g: (in milligrams) thiamine·HCl, 10; riboflavin, 15; pyridoxine·HCl, 15; Ca pantothenate, 20; folic acid, 2; niacin, 30; biotin, 0.3; inositol, 100; p-aminobenzoic acid, 25; vitamin K, 4; and tocopheryl acetate, 30; and vitamin B_{12}, 25 μg; vitamin D, 5,000 IU, and vitamin A, 20,000 IU.
[4] RSBM; or RSBM + HSBM (1:2); or HSBM; or HSBM + 0.55% inhibitor AA (thus supplying 0.11% inhibitor AA in the diet).

At the end of the trial the rats were killed, the pancreas was removed and cleaned of the accompanying fat tissue and then weighed.

RESULTS

Determination of amount of soybean trypsin inhibitors in RSBM

The trypsin- and chymotrypsin-inhibiting activities in RSBM were determined in an aqueous extract (10 g/100 ml) of finely ground RSBM. It appears that the trypsin inhibitors of RSBM are fully soluble in water; this has been proved by the fact that the second and the third aqueous extracts of RSBM contained amounts of inhibiting activity that corresponded to their calculated content in the water absorbed by the precipitated residue. It was found that 1 g RSBM contains 124 TIU and 61 ChIU.

The specific inhibiting activities per milligram of inhibitor were determined as 8.0 TIU and 10.0 ChIU for AA and as 6.0 TIU [5] and 0.75 ChIU for CSBTI. Since we have found earlier that the trypsin- and the chymotrypsin-inhibiting activities of RSBM originate mainly in these 2 inhibitors,[6] it can be calculated that 1 g of RSBM contains approximately 13.5 mg CSBTI and 5.5 mg of AA. Inhibitor AA is responsible for approximately 35% of the total trypsin-inhibiting activity and for approximately 85% of the total chymotrypsin-inhibiting activity of RSBM, whereas CSBTI is responsible for 65% of the trypsin-inhibiting activity and for 15% of the chymotrypsin-inhibiting activity of RSBM.

Influence of ethanolic extraction of RSBM on the removal from and inactivation of trypsin inhibitors in the residue

Table 3 summarizes the fate of the trypsin- and chymotrypsin-inhibiting activities of RSBM after different ethanolic extractions. As shown in table 3, the extractions performed at 55° destroyed about two-thirds of the total trypsin-inhibiting activity, whereas the loss of chymotrypsin-inhibiting activity was much smaller. This fact shows that CSBTI, which possesses a smaller chymotrypsin-inhibiting activity, either loses its activity or becomes water-insoluble by this treatment, whereas the inhibiting activity of AA remains almost unchanged.

The 60% ethanolic extraction at 55° removed more inhibiting activity than at 25° and contained only the AA inhibitor.

Extraction with 80% ethanol at 25° did not remove any inhibiting activity, but when the extraction was carried out at 55° the CSBTI was almost completely inactivated. Under all conditions no difference was found between 30 minutes and 2 hours of extraction. The temperature of 55° per se did not have any effect on either activity since aqueous extraction of RSBM at 55° for 2 hours did not cause any decrease or inactivation of either CSBTI or AA.

Influence of trypsin-inhibitor-supplemented diets on growth response of chicks and rats

The following three growth trials — two with chicks and one with rats — were carried out to study the effect of trypsin inhibitors on the growth of these living organisms.

Trial no. 1. This trial was performed with chicks. The influence of trypsin inhibitors added to the diet on growth rate, on pancreas hypertrophy, on weight gain per food consumed and on proteolytic activity in the small intestine was examined. The results are presented in table 4 and figure 1. The addition of trypsin inhibitors to a HSBM-containing diet resulted in a slight and nonsignificant decrease of growth rate, although it caused a significant hypertrophy of the pancreas. The proteolytic activity in the small intestine, as measured by the casein digestion method, was decreased by addition of AA but not influenced by CSBTI.

The diet that contained one-third of RSBM and two-thirds HSBM as protein source caused a significant decrease in growth rate, and was responsible for hypertrophy of the pancreas and for partial inhibition of the proteolytic activity in the small intestine. Of special note is the great difference in growth rate between the group that obtained RSBM+HSBM (1:2) and the group which was given HSBM+AA, although both diets contained the same trypsin-inhibiting potency in vitro, and the latter possessed 2.5 times more chymotrypsin-inhibiting activity.

Trial no. 2. In view of the results of trial no. 1, an additional factorial growth experiment with chicks was carried out. In that trial the influence of the addition of inhibitor AA to the diet on the growth response was tested at two protein levels (15% and 25%) with and without addition of 0.6% DL-methionine. The diets of two other groups of chicks had a protein level of 15% with and without addition of 0.6% DL-methionine and were supplemented with inhibitor AA in the amount of 16.5 mg (instead of 5.5 mg as usually) per g of HSBM, thus yielding diets that contained the same in vitro trypsin-inhibiting activity as the diet with RSBM.

[5] This specific activity of CSBTI was taken from the original paper by Kunitz (7). Commercial samples of CSBTI contained usually much lower (50–70%) activity.

[6] Unpublished observations by the authors.

TABLE 3

Effect of conditions of ethanolic extraction of raw soybean meal (RSBM) on trypsin-inhibiting, chymotrypsin-inhibiting, and hemagglutinating activities

Conditions of ethanolic extraction			Residue from 1 kg RSBM	Protein content of residue [1]	Activity [2]								HU [5] in residue	TIU/ChIU [6]	
					TIU [3]				ChIU [4]						
Ethanol	Temperature	Time			In residue	In extract	Total		In residue	In extract	Total			Residue	Extract
%	*degrees*	*hours*	*g*	%	*units/g RSBM*			% [7]	*units/g RSBM*			% [7]			
60	55	2	720	61.2	18.6	24.5	43.1	34.8	22.6	22.3	44.9	73.5	none	0.9	1.1
60	55	18			21.5	21.3	42.8	33.6	19.8	23.5	43.3	71.0	none	1.1	0.9
60	25	2	780	60.2	88.0	15.5	103.5	83.5	45.0	13.7	58.7	96.4	29	2.0	1.1
60	25	18			42.0	17.0	59.0	47.5	32.0	17.9	49.9	81.8		1.3	1.0
80	55	2	820	57.2	45.2	none	45.2	36.5	49.0	none	49.0	80.5	none	0.9	
80	55	18			28.9	none	28.9	23.2	37.0	none	37.0	60.7	none	0.8	
80	25	2	860	55.8	112.0	none	112.0	90.4	57.0	none	57.0	93.6	33	2.0	
80	25	18			108.0	none	108.0	87.0	55.0	none	55.0	90.2		2.0	

[1] N × 6.25. The protein content of RSBM is 49.5%.
[2] The activities were determined in aqueous extracts (10 g/100 ml) of the different meals.
[3] Trypsin inhibitor units (8, 10).
[4] Chymotrypsin inhibitor units (8, 10).
[5] Hemagglutinating units (11).
[6] The TIU/ChIU ratio in RSBM is 2.
[7] 100% corresponds to 124 TIU, 61 ChIU and 33 HU/g RSBM.

TABLE 4

Effect of diets supplemented with trypsin inhibitors on growth response of chicks

No.	Protein source [1,2]	Activity [3]		Growth rate	Wt gain/ food consumed	Pancreas wt
		TIU	ChIU			
		units/g food		*g/chick/day*		*mg/100 g body wt*
1	RSBM	62.0	30.5	3.24 [a 4]	0.24	859 [a]
2	RSBM+HSBM (1:2)	20.7	10.2	5.31 [b]	0.39	901 [a]
3	HSBM	—	—	6.99 [c]	0.50	543 [c]
4	HSBM+0.27% [5] AA	21.6	27.0	6.60 [c]	0.48	809 [a]
5	HSBM+0.27% [5] CSBTI	8.1	1.0	6.76 [c]	0.48	672 [b]

[1] Protein level 25%, 10 New Hampshire × Leghorn chicks/replicate, duration of trial 7 days.
[2] RSBM indicates raw soybean meal; HSBM, heated soybean meal.
[3] TIU indicates trypsin inhibitor units; ChIU, chymotrypsin inhibitor units.
[4] All groups not designated with the same letter are statistically different at the 5% level.
[5] Per cent in diet.

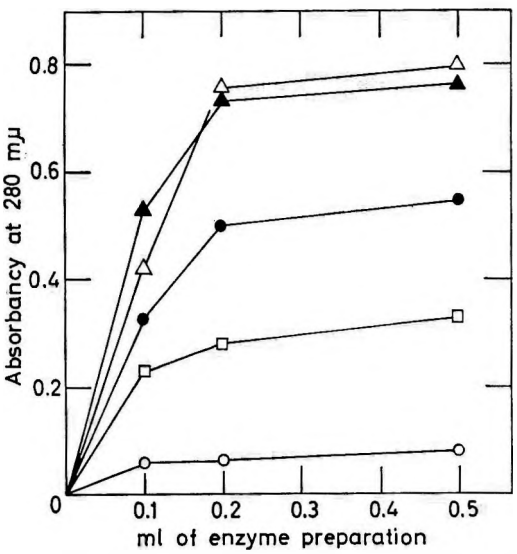

Fig. 1 Effect of soybean trypsin inhibitors added to the diets on the proteolytic activity in small intestine of chicks. The protein sources were RSBM (open circles), RSBM + HSBM in the ratio of 1:2 (solid circles), HSBM (open triangles), HSBM supplemented with 5.5 mg inhibitor AA per 1 g HSBM (open squares), and HSBM supplemented with 5.5 mg CSBTI per 1 g HSBM (solid triangles).

In this trial the total proteolytic activity in the small intestine was examined by the casein digestion method. The tryptic and chymotryptic activities of the pancreas were assayed as well. The results are shown in table 5 and figure 2.

The differences in growth rate and in the extent of the hypertrophy of the pancreas were analyzed statistically by factorial analysis of variance (14) in 2 ways: (a) factorial analysis of all the groups fed at a 15% protein level, thus yielding five different protein sources in 2 levels of methionine (zero and 0.6%); and (b) factorial analysis of 4 groups fed different soybean meals: RSBM, RSBM+HSBM (1:2), HSBM and HSBM+AA (5.5 mg/g of HSBM), every group at 2 levels of protein and 2 levels of methionine, thus giving a 3-factorial (protein source, protein level, methionine) 4×2×2 experiment.

The results of the analyses of the growth rate differed slightly by the two different statistical methods of analysis. With a protein level of 15% the main effect, that is, protein source and methionine, was highly significant ($P < 0.01$) but no significant interaction was noted.

Significant differences ($P = 0.05$) were found between the group given RSBM and RSBM+HSBM (1:2) and the others, but the differences between HSBM, HSBM+0.16% AA and HSBM+0.48% AA were not statistically significant. In the 3-factorial analyses all 3 main effects, that is, protein source, protein level and methionine, were highly significant ($P < 0.01$) and the 4 protein sources were found to be significantly different ($P = 0.05$) from each other. In these analyses a significant interaction was found between the different protein sources and addition of methionine. As shown in table 5 the order of growth response to different protein sources remains the same at all levels of protein and methionine, but the relative growth rate as compared with HSBM varies in diets containing RSBM and RSBM+HSBM (1:2) with the addition of methionine.

TABLE 5

Effect of addition of trypsin inhibitors to diets of different levels of protein and methionine on growth response of chicks

No.	Protein level	Methionine added	Protein source [1,2]	Activity [3]		Growth rate	Wt gain/ food consumed	Pancreas wt	Activity in intestine [4]	
				TIU	ChIU				Trypsin	Chymo-trypsin
	%	%		*units/g food*		*g/chick/day*		*mg/100 g body wt*	*units/mg chyme*	
1	15	none	RSBM	37.2	18.3	0.80	0.08	865	0.32	1.23
2	15	none	RSBM+HSBM (1:2)	12.4	6.1	1.96	0.19	830	1.39	3.42
3	15	none	HSBM	—	—	4.88	0.31	645	2.34	3.13
4	15	none	HSBM+0.16% [5] AA	12.8	16.0	4.72	0.37	781	0.81	1.61
5	15	none	HSBM+0.48% [5] AA	38.4	48.0	3.81	0.29	853	0.47	0.76
6	15	0.6	RSBM	37.2	18.3	2.21	0.20	1020	0.36	0.47
7	15	0.6	RSBM+HSBM (1:2)	12.4	6.1	4.00	0.32	966	1.04	3.25
8	15	0.6	HSBM	—	—	5.56	0.37	650	2.45	4.23
9	15	0.6	HSBM+0.16% [5] AA	12.8	16.0	4.79	0.37	899	0.69	2.60
10	15	0.6	HSBM+0.48% [5] AA	38.4	48.0	4.49	0.34	1066	0.69	1.12
11	25	none	RSBM	62.0	30.5	1.46	0.13	809	0.12	0.42
12	25	none	RSBM+HSBM (1:2)	20.7	10.2	4.12	0.32	991	0.63	3.10
13	25	none	HSBM	—	—	6.53	0.47	629	2.88	4.94
14	25	none	HSBM+0.27% [5] AA	21.6	27.0	5.68	0.42	975	0.47	1.67
15	25	0.6	RSBM	62.0	30.5	3.22	0.26	886	0.16	0.32
16	25	0.6	RSBM+HSBM (1:2)	20.7	10.2	4.99	0.34	988	0.73	2.63
17	25	0.6	HSBM	—	—	7.06	0.45	673	2.56	5.52
18	25	0.6	HSBM+0.27% [5] AA	21.6	27.0	5.92	0.41	957	0.66	1.55
19	25	0.6	HSBM+0.27% [5] CSBTI	8.1	1.0	6.39	0.45	818	3.17	9.52

[1] 10 New Hampshire × Leghorn chicks/replicate, duration of trial 7 days. The details of statistical analysis are given in text.
[2] RSBM indicates raw soybean meal; HSBM, heated soybean meal; CSBTI, crystalline Kunitz soybean trypsin inhibitor units; AA, trypsin and chymotrypsin inhibitor AA.
[3] TIU indicates trypsin inhibitor units; ChIU, chromotrypsin inhibitor units.
[4] One unit corresponds to increase in $10^{-3}\times$ extinction at 253 mμ for trypsin and decrease in $10^{-3}\times$ extinction at 233 mμ for chymotrypsin/one minute of reaction at 25°.
[5] Per cent in diet.

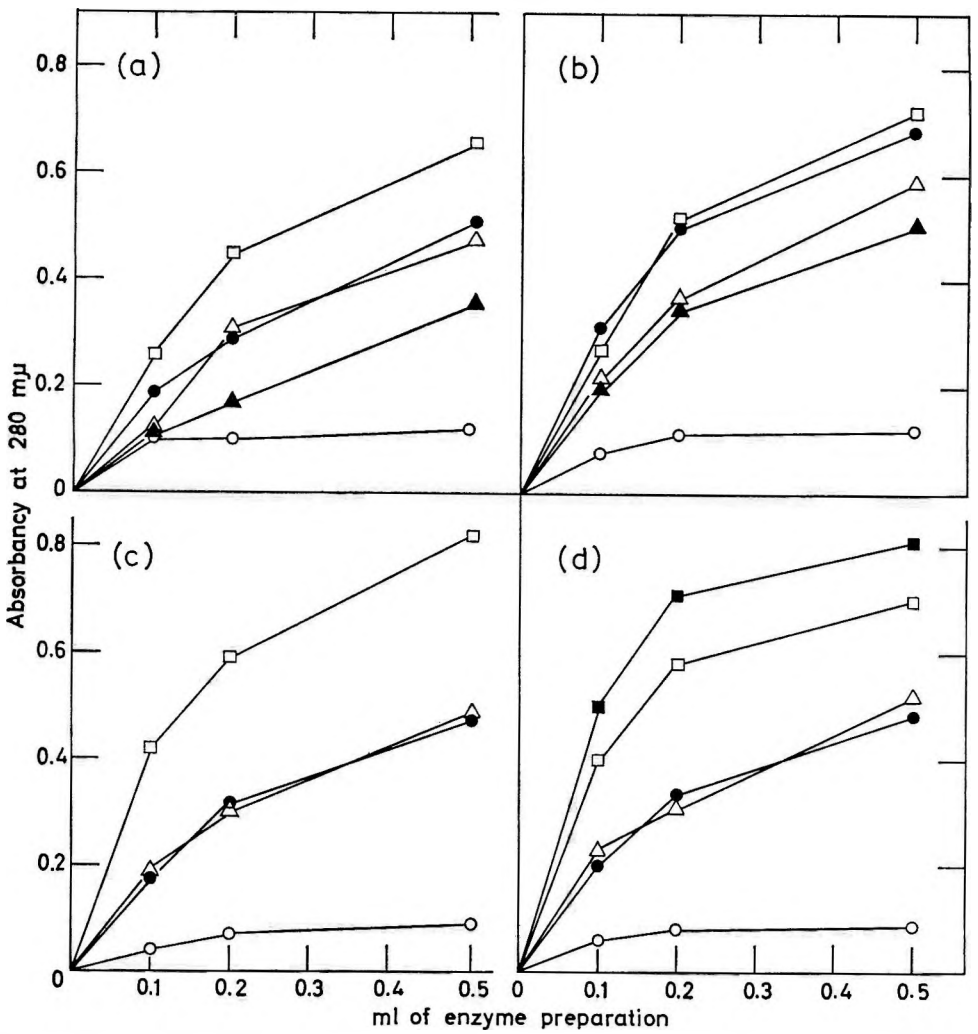

Fig. 2 Effect of addition of soybean trypsin inhibitors to diets with different protein and methionine levels on the proteolytic activity in the small intestine of chicks. (a) Protein level 15%, no methionine added; (b) protein level 15%, 0.6% methionine added. (c) protein level 25%, no methionine added; and (d) protein level 25%, 0.6% methionine added. The protein sources were RSBM (open circles), RSBM + HSBM in the ratio of 1:2 (solid circles), HSBM (open squares), HSBM supplemented with 5.5 mg CSBTI per 1 g HSBM (solid squares), HSBM supplemented with 5.5 mg inhibitor AA per 1 g HSBM (open triangles) and HSBM supplemented with 16.5 mg inhibitor AA per 1 g HSBM (solid triangles).

The statistical analyses of differences in pancreas hypertrophy caused by different diets were carried out in a similar way. Highly significant main effects of methionine and protein source were found by both methods of statistical analysis. The addition of methionine increased the pancreas hypertrophy mainly in the lower level of protein, whereas in the higher level of protein the effect was much smaller. No significant differences were found between the following protein sources: RSBM, RSBM+HSBM (1:2) and HSBM+AA in two AA levels, but the effects of all these diets differed significantly from that of HSBM.

The diet which contained HSBM supplemented by CSBTI was compared by

TABLE 6

Effect of diets supplemented with inhibitor AA on growth response of rats

Sex	Protein source [1,2]	Activity [3]		Growth rate	Wt gain/ food consumed	Pancreas wt
		TIU	ChIU			
		units/g food		*g/rat/day*		*mg/100 g body wt*
Males	RSBM	24.8	12.2	1.87 [a 4]	0.16	725 [a 4]
	RSBM+HSBM (1:2)	8.3	4.1	2.95 [b]	0.23	625 [ab]
	HSBM	—	—	3.66 [c]	0.28	436 [c]
	HSBM+0.11% [5] AA	8.8	11.0	3.28 [bc]	0.27	541 [b]
Females	RSBM	24.8	12.2	1.37 [a]	0.14	783 [a]
	RSBM+HSBM (1:2)	8.3	4.1	2.54 [b]	0.21	687 [ab]
	HSBM	—	—	3.40 [c]	0.26	431 [c]
	HSBM+0.11% [5] AA	8.8	11.0	2.75 [b]	0.23	599 [b]

[1] Protein level 10%, duration of trial 14 days.
[2] RSBM indicates raw soybean meal; HSBM, heated soybean meal.
[3] TIU indicates trypsin inhibitor units; ChIU, chymotrypsin inhibitor units.
[4] All groups not designated with the same letter are statistically different at the 5% level.
[5] Per cent in diet.

Student's *t* test (14) with HSBM at a protein level of 25% supplemented with methionine. As in trial no. 1, a small but nonsignificant decrease in growth rate was found, but a significant hypertrophy of the pancreas was caused by the CSBTI-supplemented diet.

As shown in figure 2, similar proteolytic activities in the small intestine were found at all levels of protein and methionine and the differences in proteolytic activity were caused mainly by the variety in protein source. The trypsin and chymotrypsin activities in the intestine (table 5) are similar to those shown in figure 2, although generally the comparative trypsin activity was inhibited to a greater extent than the chymotrypsin. The addition of CSBTI increased the proteolytic activity of both enzymes in the intestine (fig. 2 and table 5).

Trial no 3. Since trials 1 and 2 showed only a small inhibiting effect of inhibitor AA on the growth rate of chicks, a comparative experiment with rats was carried out. The trial was performed with males and females as described above. The results are presented in table 6.

Similar results were obtained with males and females and no interaction between the sex and protein source was found. Similar to the growth experiments with chicks, the growth of rats was impaired only slightly by AA-supplemented HSBM diets, although a significant hypertrophy of the pancreas was noted as compared with that caused by HSBM diets without AA.

Determination of nutritional value and effect on pancreas of ethanol-extracted soybean meals by growth trials with chicks

Trials 4, 5, 6 and 7 were carried out on chicks fed EERSBM-supplemented diets. The effect of these protein sources on the rate of growth, on weight gain per food consumed, on the pancreas hypertrophy and on the proteolytic activity in the small intestine were studied.

In trials 4 and 5, EERSBM prepared by extraction with 60% ethanol at 25° served as the only protein source, and in trials 6 and 7, EERSBM, prepared by extraction with 60% ethanol at 55°. Both diets were compared with the corresponding RSBM diets. The results are presented in table 7 and in figure 3.

As shown by trials 4 and 5, EERSBM prepared by extraction with 60% or 80% ethanol at 25° was responsible for a higher, although not statistically significant, growth rate than RSBM. Extraction with 60% ethanol removes only about 15% of total trypsin-inhibiting activity, whereas with 80% ethanol no inhibiting activity was removed at all. The two experimental meals caused a significant hypertrophy of the pancreas, practically the same as with RSBM, and inhibited likewise the proteolytic activity in the intestine.

As shown by trials 6 and 7, the nutritional value of EERSBM, prepared by extraction with 60% or 80% ethanol at 55°,

TABLE 7

Effect of ethanol-extracted raw soybean meals (EERSBM) [1] *on growth response and pancreas hypertrophy of chicks*

Trial no.	Protein source [2]	Conditions of ethanolic extraction	Activity [3]		Growth rate	Wt gain/ g food consumed	Pancreas wt
			TIU	ChIU			
			units/g food		*g/chick/day*		*mg/100 g body wt*
4 [4]	RSBM	60% EtOH, 25°	42.1	20.8	1.59 [a 5]	0.25	732 [a]
	EERSBM		36.6	17.3	1.95 [a]	0.32	762 [a]
	EERSBM	80% EtOH, 25°	46.0	23.2	1.84 [a]	0.28	757 [a]
	HSBM		0	0	3.82 [b]	0.52	483 [b]
5 [6]	RSBM		54.6	27.0	2.72 [a]	0.26	912 [a]
	EERSBM	80% EtOH, 25°	59.6	30.2	3.47 [a]	0.36	908 [a]
	HSBM		0	0	5.23 [b]	0.46	663 [b]
6 [7]	RSBM		54.6	27.0	2.15 [a]	0.24	919 [a]
	EERSBM	60% EtOH, 55°	5.8	2.5	3.96 [c]	0.44	662 [b]
	EERSBM	80% EtOH, 55°	28.1	27.7	3.07 [b]	0.36	852 [a]
	HSBM		0	0	4.98 [d]	0.46	615 [b]
7 [8]	RSBM		62.0	30.5	3.39 [a]	0.29	849 [a]
	EERSBM	60% EtOH, 55°	6.5	2.9	6.08 [c]	0.44	650 [b]
	EERSBM	80% EtOH, 55°	31.9	31.4	5.35 [b]	0.40	757 [a]
	HSBM		0	0	7.97 [d]	0.56	417 [c]

[1] Residues after 2 hours' ethanolic extraction of RSBM under condition listed within table.
[2] RSBM indicates raw soybean meal; HSBM, heated soybean meal.
[3] TIU indicates trypsin inhibitor units; ChIU, chymotrypsin inhibitor units.
[4] Protein level 17%, 10 Leghorn chicks/replicate, duration of trial, 7 days.
[5] All groups not designated with the same letter (within the trial) are statistically different at 5% level.
[6] Protein level 22%, 10 Leghorn chicks/replicate, duration of trial, 10 days.
[7] Protein level 22%, 20 Leghorn chicks/replicate, duration of trial, 10 days.
[8] Protein level 25%, 20 New Hampshire chicks/replicate, duration of trial, 10 days.

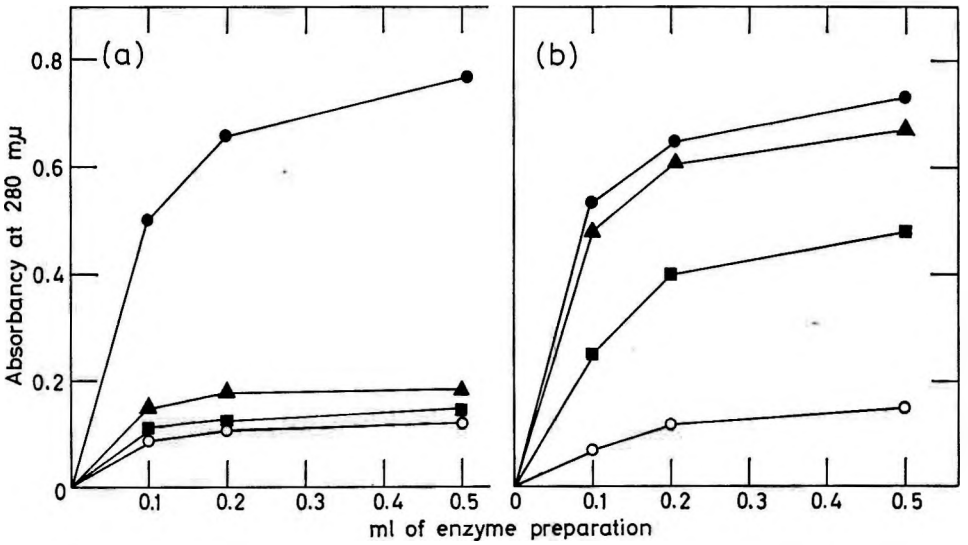

Fig. 3 Effect of ethanol-extracted raw soybean meal, as protein source in the diet of chicks, on the proteolytic activity in the small intestine. (a) Extraction was performed at 25°; (b) extraction was performed at 55°. The protein sources were RSBM (open circles), HSBM (solid circles), RSBM extracted with 80% ethanol (solid squares), and RSBM extracted with 60% ethanol (solid triangles).

is significantly superior to that of RSBM. Extraction with 80% ethanol removed or destroyed approximately 50% of the trypsin-inhibiting activity, and extraction with 60% ethanol, about 90%. As shown in table 7 by trials 6 and 7, EERSBM extracted with 60% ethanol at 55° caused only a small pancreas hypertrophy, and no inhibition of proteolytic activity in the small intestine was noted, but the growth rate was significantly lower than that caused by the HSBM diet.

Ethanol-extracted soybean meal, prepared by extraction with 80% ethanol at 55°, caused a greater pancreas hypertrophy, a lower growth rate and a partial inhibition of the proteolytic activity in the small intestine as compared with EERSBM prepared by 60% ethanol at 55°, but was significantly different from RSBM.

Growth trials with chicks fed the HSBM diet supplemented with the insoluble dry residue of 80% ethanolic extract (prepared at 25° or 55°) as the only protein source, showed no difference in growth rate, in pancreas hypertrophy nor in proteolytic activity in the small intestine as compared with the chicks fed HSBM diets. (The 80% ethanolic extract contained no trypsin- or chymotrypsin-inhibiting activities.)

DISCUSSION

The results of the experiments with the two pure and different soybean trypsin inhibitors (7–9) demonstrate clearly that both of them cause a pronounced pancreatic hypertrophy in chicks and rats (table 5). This hypertrophy is accompanied only by a slight growth depression of the animals, as also observed in other investigations with partially purified preparations or with the Kunitz (7) crystalline soybean trypsin inhibitor (15, 16). This substantiates the assumption that pancreatic hypertrophy caused by trypsin inhibitors does not imply animal growth depression. The observations of Saxena et al. (5) that pancreatic hypertrophy is caused by soybean meal fractions devoid of trypsin-inhibiting activity does not rule out the possibility that other soybean proteins are also able to produce this phenomenon.

A pronounced difference between inhibitor AA and CSBTI was noted in their effect on the proteolytic activity in the small intestine. CSBTI-supplemented diets did not cause any inhibition of the proteolytic activity in the small intestine whereas AA did. This difference may be attributed to the following: (a) CSBTI possesses only approximately 8% of the specific activity of AA toward chymotrypsin (9); (b) CSBTI is generally less stable than AA as indicated by its inactivation while incubated in acidic medium or with pepsin (8, 17), as well as by the varying activities of the crystalline commercial preparations [7]; and (c) CSBTI was given in a smaller amount than AA on an activity basis (TIU/g diet).

The comparative effects of the addition of pure soybean trypsin inhibitors on growth, as expressed in the various growth experiments, are summarized in the form of an index in table 8. The HSBM diet was taken as 100. The growth impairment caused by trypsin inhibitors added to HSBM diets was rather small as compared with diets that contained RSBM or RSBM —HSBM (1:2) and had the same range of trypsin-inhibiting activity. The RSBM contains, possibly, another heat-labile factor(s), which also remains in the RSBM residue after extraction at pH 4.2 (6), and causes growth impairment. This possibility was pointed out previously by Hayward et al. (18), who concluded that RSBM contains some "unavailable essential protein factor," and was reported again by Lepkovsky et al. (19) on the basis of their study of intestinal proteolysis of chicks given RSBM and HSBM meals for different lengths of time. These investigators have pointed out that such "unavailable protein" can be also formed in the intestine through trypsin inhibitors from the raw soybean meal, the endogeneous protein or both, which is in agreement with the observations of Bielorai and Bondi (20), and of de Muelenaere (21). The possibility, however, that the combination of the 2 inhibitors or their association with other proteins in the intact raw meal is responsible for growth impairment, should not be ruled out.

Two inhibitory, growth-impairing mechanisms resulting from RSBM in the diet may therefore be suggested.

[7] See footnote 5.

TABLE 8

Index of comparative influence of trypsin inhibitors on growth rate of chicks and rats [1]

Protein source [2] in diet	Trial no.						
	1 [3]	2 [4]	2 [5]	2 [6]	2 [7]	3 [8]	3 [9]
HSBM	100	100	100	100	100	100	100
HSBM+CSBTI [10]	97	91	—	—	—	—	—
HSBM+AA [10]	94	84	87	86	96	90	81
HSBM+AA [11]	—	—	—	81	78	—	—
HSBM+RSBM (2:1)	76	71	62	72	40	80	75
RSBM	46	41	22	40	16	51	40

[1] Results are taken from tables 4, 5 and 6.
[2] HSBM indicates heated soybean meal; CSBTI, crystalline Kunitz soybean trypsin inhibitor units; RSBM, raw soybean meal; AA, inhibitor AA.
[3] Chicks, protein level 25%, supplemented with methionine.
[4] Chicks, protein level 25%, supplemented with methionine.
[5] Chicks, protein level 25%, no methionine supplement.
[6] Chicks, protein level 15%, supplemented with methionine.
[7] Chicks, protein 15%, no methionine supplement.
[8] Male rats, protein level 10%, supplemented with methionine.
[9] Female rats, protein level 10%, supplemented with methionine.
[10] 5.5 mg inhibitor/g HSBM.
[11] 16.5 mg inhibitor/g HSBM.

(a) The trypsin inhibitors stimulate pancreatic hypertrophy and synthesis of proteolytic enzymes, thus increasing the requirements for amino acids and loss of endogenous nitrogen. Furthermore, the trypsin inhibitors inhibit directly the proteolysis in the small intestine, especially shortly after ingestion and digestion of RSBM, before the increased synthesis and secretion of pancreatic enzymes is achieved (19, 22, 23). The fact that the inhibition of proteolysis is found mainly in chicks but not in rats and mice ((24) and fig. 1) may be attributed to the loss of trypsin-inhibiting activity of CSBTI due to the peptic digestion in rats and mice (6, 8, 17).

(b) RSBM contains a protein fraction, which becomes digestible only after heating, as may be deduced from indirect evidence such as lower digestibility of RSBM (19, 25, 26) or greater growth impairment caused by RSBM diets as compared with HSBM diets supplemented with inhibitors (table 8). This possibility should not be ruled out even when the assumption concerning the presence of an additional, hitherto unknown, growth-impairing factor in RSBM is verified.

It appears that mechanism *a* plays a rather small role as compared with mechanism *b*. This is more obvious for chicks than for rats as the growth-impairing effect of inhibitor AA was more pronounced on rats than on chicks.

Methionine supplementation (table 8, see also trial 2) did not change the relative growth impairment caused by HSBM+AA diets, whereas the addition of methionine to RSBM diets, especially to those of lower protein content, decreased the relative growth impairment. It should be noted that the apparent digestibility of methionine in raw and heated soybean meal for rats (25) and chicks (26) is similar to that of total nitrogen; however, since the digestibility of RSBM is lower than that of HSBM (19, 25, 26) the methionine, which is the most-limiting amino acid in soybean meal, becomes especially critical in RSBM.

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Mobilization of Liver Vitamin A in Sheep [1,2]

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ABSTRACT Six ram lambs were injected intravenously with vitamin A acetate labeled with tritium. Liver was sampled by aspiration biopsy after 5, 33 and 61 days and then at 14-day intervals until 187 days after injection. Jugular blood was sampled frequently for the first 5 days and at the time of each liver biopsy. Feces and urine were collected for the first 5 days and for a 24-hour period immediately prior to each liver biopsy. Ether extracts of serum collected 5 minutes after dosing accounted for only 5.16% of the injected radioactivity. Liver vitamin A reserves decreased from 260 μg/g of liver at 5 days to 89 μg/g at 89 days. Radioactivity decreased a proportionate amount during this period. Depletion from the liver was then stopped by vitamin A supplementation. A half-time of 75 days was calculated from the decrease in the specific activity of liver vitamin A between 89 and 187 days. Detection of radioactivity in all blood samples supported the conclusion that there was continuous turnover of vitamin A stores. Ether extracts of fecal samples contained traces of radioactivity. Radioactivity was not detected in urine.

Since the liver is the primary storage site for vitamin A, liver vitamin A stores are widely used to evaluate the vitamin A status of animals and their ability to compensate for a dietary deficiency. An improved understanding of the processes involved in the deposition and removal of vitamin A from the liver would increase the usefulness of such evaluations. Several workers have studied changes in vitamin A stores during periods of vitamin A depletion or following vitamin A therapy (1); however, little attention has been given to the stability of vitamin A reserves when total vitamin A storage is not changing. The results of preliminary experiments conducted at this station suggest continuous removal of vitamin A from the livers of rats fed adequate carotene and vitamin A to maintain total stores of vitamin A at a constant level. The experiment reported here was designed to extend this observation to sheep and to provide an estimate of turnover time.

EXPERIMENTAL PROCEDURE

Six crossbred ram lambs, averaging 32 kg, were fed green leafy alfalfa hay for 60 days before each received an intrajugular injection of 4.8 mg of vitamin A acetate labeled with 384 uCi of tritium attached to the carbons on either side of the second double bond from the ester end [4] suspended in 4 ml of 20% aqueous polyoxyethylene sorbitan monooleate (Tween 80) by the procedure of Bieri (2). Although the sheep were fed ground alfalfa hay ad libitum, a continuous decrease in liver vitamin A stores occurred during the first 75 days after treatment. In order to stop this depletion, ground shelled corn supplemented with vitamin A palmitate was added to the hay. A daily allowance of 726 g of corn/sheep supplied 770 μg of the supplemental vitamin A, which was about 150% of NRC (3) recommendations.

Jugular blood was sampled 5, 15 and 30 minutes and 1, 3 and 6 hours after injection of the labeled vitamin A acetate. The first 4 samples were taken from the jugular vein that had not received the injection. Subsequent blood samples were collected once daily for the first 5 days and at the time of each liver biopsy. Feces and urine were collected and sampled daily for the first 5 days and for a 24-hour period immediately prior to each liver biopsy.

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[4] Prepared and donated by Hoffmann-La Roche, Inc., Nutley, New Jersey.

Liver samples were taken by the aspiration biopsy technique of Dick (4) 5, 33 and 61 days after treatment, and then at 14-day intervals until the study was terminated. Samples were stored at − 20° until they were analyzed.

Serum was extracted by the method of Kimble (5). Liver and fecal samples were saponified and extracted as described by Gallup and Hoefer (6). Vitamin A in the extracts was determined with trifluoroacetic acid as described by Dugan et al. (7). Radioactivity determinations were made with a liquid scintillation counter.[5] Duplicate 1-ml aliquots of petroleum ether extracts were combined with 15-ml portions of a scintillator solution containing 4 g 2,5-diphenyloxazole (PPO) and 50 mg 1,4-di 2-(5-phenyloxazolyl)-benzene (POP OP) per liter of toluene. Larger volumes of low activity extracts were evaporated to dryness and redissolved in 1 ml of petroleum ether. Urine was assayed for radioactivity by the method of Langham et al. (8). Background determinations were made with reagent blanks, and counting efficiencies were determined by adding tritiated toluene to the samples and recounting.

Separations of vitamin A alcohol and vitamin A ester were conducted by the procedure of Thompson et al. (9).

RESULTS AND DISCUSSION

Recovery of tritium activity from ether extracts of periodic serum samples is presented in table 1. When total blood volume was estimated as described by Dukes (10), radioactivity in the serum 5 minutes after injection represented 5.16% of the dose. This diminished to 0.31% by the fifth day. Rapid disappearance of vitamin A acetate injected intravenously into calves, rats and rabbits was noted by Kon et al. (11) and was confirmed in rats and rabbits by Pollard and Bieri (12). Disappearance of vitamin A from the blood could have resulted from its removal by the liver, its use by tissues, or its destruction. Pollard and Bieri (12) reported that up to one-half of the vitamin A acetate injected into rats was destroyed within 5 minutes. In studying possible mechanisms for this destruction, they found that hemolyzed blood cells would destroy vitamin A in vitro.

TABLE 1

Radioactivity in serum of sheep following intravenous injection of labeled vitamin A [1]

Time after treatment	Tritium activity
	dpm/ml
5 minutes	28,659
30 minutes	21,771
1 hour	14,058
1 day	6,071
5 days	1,419
33 days	297
61 days	274
75 days	130
89 days	394
103 days	66
117 days	61
131 days	96
145 days	33
159 days	232
172 days	48
187 days	33

[1] 384 μCi in 4.8 mg of tritium-labeled vitamin A acetate.

Only 70% of the activity in serum samples could be recovered from alumina columns. When compared with 90 to 98% recoveries for both vitamin A and tritium activity from chromatographed liver extracts, this low recovery suggests that an appreciable portion of the radioactivity in ether extracts of serum samples was associated with molecules other than vitamin A esters and vitamin A alcohol. About 83% of the activity recovered from the column was associated with the alcohol fraction within 5 minutes. This rapid appearance of the recovered activity in the alcohol fraction suggests rapid hydrolysis of vitamin A acetate.

No radioactivity could be detected in ether extracts of urine samples, which agrees with the report that no vitamin A is present in the urine of rats (14). An attempt to measure total radioactivity of the urine by the method of Langham et al. (8) was not successful. Counts were erratic and counts of urine from untreated sheep were equal to or in excess of sample counts. Traces of radioactivity were detected in ether extracts of only a few fecal samples.

Data on storage of vitamin A and radioactivity in the liver are shown in table 2. Storage of tritium activity in the liver was

[5] Baird Atomic Model 745.

TABLE 2

Vitamin A and radioactivity in livers of sheep after injection with labeled vitamin A [1]

Days after treatment	Vitamin A	Tritium activity
	μg/g	*10^2 dpm/g*
Period of vitamin A depletion		
5	260 ± 59 [2]	299 ± 30
33	203 ± 43	223 ± 11
61	124 ± 30	143 ± 17
Period of vitamin A supplementation		
75	95 ± 29	93 ± 4
89	89 ± 17	93 ± 10
103	89 ± 16	75 ± 3
117	91 ± 36	59 ± 11
131	105 ± 40	64 ± 5
145	65 ± 16	51 ± 3
159	75 ± 70	37 ± 10
172	70 ± 13	40 ± 15
187	66 ± 22	28 ± 5

[1] 384 μCi in 4.8 mg of tritium-labeled vitamin A acetate.
[2] Means ± SE.

adequate for the study of possible turnover; however, there was a marked and sustained depletion of vitamin A from 5 to 89 days after treatment. Tritium activity decreased at a similar rate. Stress from liver biopsies and confinement to metabolism crates may have contributed to the rapid depletion of liver vitamin A stores. Since the sheep were receiving high quality alfalfa hay, the depletion suggests impairment in their ability to convert carotene to vitamin A but could have been caused by carotene destruction prior to feeding.

Supplementation with ground shelled corn and vitamin A was started 68 days after treatment in order to stop depletion of vitamin A from the liver. A stabilization period of 21 days was allowed and the data collected between 89 and 187 days were selected for the estimation of turnover rate. Although the levels of vitamin A in the liver during the first and second half of the vitamin A supplementation period (table 2) are not significantly different, an apparent decrease in liver vitamin A occurred between day 131 and day 145. Reasons for this decline are not evident. It may have resulted from errors inherent in sampling from a local area of the liver by biopsy or to differences in vitamin A storage in different areas of the liver. The apparently continuous decrease in tritium activity in the liver and the detection of tritium activity in the blood throughout the trial, provide strong evidence for the continuous turnover of vitamin A stores.

Problems in estimating turnover which result from the apparent reduction in vitamin A stores between 131 and 145 days are minimized by using specific activity units (dpm/μg vitamin A).

The significant ($P < 0.01$) linear regression of the log of specific activity with time is illustrated in figure 1. Specific activity of vitamin A in the liver is plotted on a logarithmic scale against time. Zero day on the time scale represents the beginning of the turnover study or day 89 of the experiment. The regression line gives an estimated half-time of 75 days or a turnover time of 108 days (13).

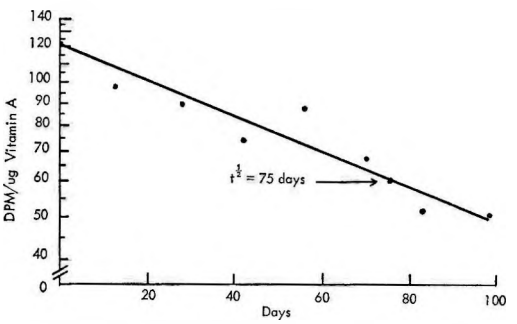

Fig. 1 Specific tritium activity of liver vitamin A stores plotted on a logarithmic scale against time (day 89 through day 187).

These data demonstrate turnover of vitamin A stores beyond that inherent in their accumulation or depletion. Under the conditions of this experiment the half-time of vitamin A in the liver was 75 days. Literature values suitable for direct comparison with this determination are not available. Similar determinations for sheep under different experimental conditions and for other species should be of considerable interest.

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Dietary ^{90}Sr Reductions through Food Substitutions in the Fruit and Vegetable Category [1]

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ABSTRACT Management of dietary practice to minimize intake of ^{90}Sr by the human population requires knowledge of the relative ^{90}Sr and calcium contributions of different foodstuffs. An analysis is presented for fruits and vegetables, a category generally neglected. The extent of reduction possible depends on the nature of the diet and the degree of constraint imposed; for example, the maximal effect from modifications in patterns of consumption and routine processing of fruits and vegetables occurs with a diet of minimal dairy products and amounts to about a 40% reduction in ^{90} Sr/Ca intake. Information of this type can be used in conjunction with other practices designed to reduce radionuclide intake. This would permit an overall assessment of the potential reductions from dietary decontamination procedures.

The pathways of ^{90}Sr in the food chain, the incorporation of ^{90}Sr into man and the subsequent potential hazards have been investigated widely (1, 2). Networks of monitoring stations have been developed which give approximations of ^{90}Sr intake from individual foods and total diets.[2] Foods have generally been considered in 4 categories as far as ^{90}Sr contribution is concerned: dairy products, grain products, fruits and vegetables, and meat, fish and eggs. Most emphasis has been given to dairy products because they have been the largest contributors of calcium and therefore ^{90}Sr to human diets in countries such as the United States where relatively large quantities of milk are consumed. Only small amounts of spotty data are available for fruits and vegetables; this is primarily because of the large number of individual products and sampling difficulties that must be taken into account.

The present paper treats in detail the role of fruits and vegetables as contributors of ^{90}Sr. Information of this type may be useful for assessment of risk to special population groups or for those who may follow dietary habits that tend to increase dietary levels of ^{90}Sr/Ca (for example, high plant food intake, minimal milk consumption, and minimal processing of foods). Such groups should be among the first to be considered following incidents that produce increased environmental radiocontamination. Estimates are presented of the reductions of ^{90}Sr intake that can be obtained by practical shifts of consumption within the fruit and vegetable category, with due attention being given to maintenance of nutritional balance.

The Federal Radiation Council has published a series of staff reports on background material for the development of radiation protection standards.[3] They range in scope from the development of protective action guides for specific radionuclides to estimates of fallout levels and the health implications of such fallout. The Federal Radiation Council has concluded "that the health risk from radioactivity in food over the next several years would be too small to justify protective actions to limit the intake of radionuclides either by diet modifications or by altering the normal distribution and use of food, particularly milk and dairy products" (3). They recommend that surveillance of the radionuclide content in food products be continued at levels appropriate to the situation. The Federal Radiation Council has also recognized that long-term changes in agricultural practices, food

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[1] Supported under contract with the U. S. Atomic Energy Commission.
[2] Details of these networks can be found in: Radiological Health Data and Reports, vol. 1, 1960 through vol. 7, 1966. Public Health Service, Washington, D. C.; and in Health and Safety Laboratory Fallout Program Reports, HASL-88, 1960 through HASL-172, 1966. U.S. Atomic Energy Commission, New York Operations, New York.
[3] Federal Radiation Council, Staff Reports no. 1, 1960 through no. 7, 1965. Superintendent of Documents, U.S. Government Printing Office, Washington, D. C.

processing and dietary habits could bring about reductions in the transmission of radionuclides to man but the annual radiation dose from environmental contamination does not warrant such actions. If protective actions should become necessary, the consideration of specific food categories such as fruits and vegetables would form an important part of any remedial measures.

METHODS

The proper nutrient or dietary balance is governed by the amounts of energy, protein, vitamins and minerals. There is generally little concern for providing sufficient calories if the diet is otherwise properly balanced. Fruits and vegetables are most important as contributors of vitamin A and ascorbic acid to the diet. Thus, any reduction in dietary ^{90}Sr intake by substitutions within this category should be accompanied by a maintenance of the levels of vitamin A and ascorbic acid. Under emergency conditions supplemental vitamin sources could be utilized if more drastic intake revisions were to be considered.

Analytical values of ^{90}Sr were used as reported by the Food and Drug Administration for the raw, unwashed and unprocessed product (4–8). As values for calcium were not given, the individual comparisons are made on a basis of ^{90}Sr intake and conversions to a $^{90}Sr/Ca$ basis are made later when considering relationships to the total diet. It was necessary to average values over the 3-year period 1961–63 because in no single year were enough samples of different products taken to permit detailed analyses. Even though the nature of contamination was different in these years, the relative ranking of the various products as to ^{90}Sr content was in reasonable agreement among the three sample periods. It was considered, therefore, that 3-year averages were satisfactory for the purposes of the study.

The major fruits and vegetables were ranked according to their ^{90}Sr contributions with values also given for vitamin A and ascorbic acid (tables 1 and 2). Average ^{90}Sr levels for the 1961–63 period were used with average per captita consumption data to calculate the estimated annual ^{90}Sr intake for each item (4–10). Data for vitamin intake were obtained in the same manner by using food composition tables (11). The fruits and vegetables included in these computations represent 85% of the total fruit and vegetable consumption. The major ^{90}Sr contributors were oranges, potatoes, lettuce, grapes and nuts. The primary sources of vitamins were cantaloupes, peaches, citrus, carrots, sweet potatoes, tomatoes, potatoes and spinach.

Two considerations were taken into account for estimation of the effect of possible ^{90}Sr reductions in the fruit and vegetable category: processing, and the

TABLE 1

^{90}Sr, vitamin A and ascorbic acid intake of major fruits, berries and melons in the United States, three-year average 1961–63

	Per capita consumption	Estimated annual intake		
		^{90}Sr	Vitamin A	Ascorbic acid
	kg/year	*pCi/year*	*IU/year*	*mg/year*
Oranges	12.5	96	25,000	6,240
Grapes	2.3	67	2,300	90
Nuts	0.7	59	200	15
Peaches	6.9	42	89,600	480
Apples	10.9	38	9,800	430
Watermelons	7.1	22	42,000	500
Strawberries	1.3	22	800	780
Cranberries	0.5	16	200	60
Pears	2.2	13	400	90
Cantaloupe	3.9	11	132,000	1,280
Grapefruit	8.4	9	6,700	3,200
Cherries	1.0	6	9,500	100
Pineapple	2.7	4	1,900	460
Plums and prunes	1.4	2	15,600	30
Apricots	0.8	1	21,300	80

TABLE 2

^{90}Sr, vitamin A and ascorbic acid intake of major vegetables in the United States, three-year average 1961–63

	Per capita consumption	Estimated annual intake		
		^{90}Sr	Vitamin A	Ascorbic acid
	kg/year	*pCi/year*	*IU/year*	*mg/year*
Leafy vegetables:				
Lettuce	9.4	73	30,900	560
Spinach	1.0	38	79,400	500
Cabbage	5.0	30	6,500	2,350
Celery	3.3	25	8,000	300
Cauliflower	0.9	3	500	690
Kale	0.1	3	5,700	100
Broccoli	0.4	3	11,000	510
Podded, root and other vegetables:				
Potatoes	47.3	94	tr	9,500
Sweet potatoes	3.2	46	279,000	670
Tomatoes	13.3	35	120,000	3,060
Carrots	3.7	30	408,100	300
Onions	5.3	25	13,300	580
Green beans	3.4	24	20,600	650
Lima beans	0.6	2	1,800	180
Other beans, dried	3.5	14	350	tr
Peas, green	2.8	16	19,000	590
Cucumbers	3.4	12	8,400	30
Beets	0.9	8	180	90
Peppers	1.2	1	4,900	1,500
Pumpkin and squash	0.3	1	3,300	50
Asparagus	0.7	1	6,200	230
Artichokes	0.1	1	180	10

balancing of ^{90}Sr and vitamin contributors. Since many of these items are consumed in fresh form and the degree of preparation is unknown, it was necessary to determine the total reductions possible through processing practices. Therefore, the quantities of fruits and vegetables normally consumed in fresh form were converted to processed-form equivalents. In this conversion process the effects of standard processing practices on ^{90}Sr intake totals would reflect the intake from an "all-processed" consumption pattern. The nature and magnitude of the ^{90}Sr reduction factors used in this conversion were obtained from studies conducted under actual commercial operations (6, 7, 12). For those items in which no appropriate reduction factor was available an average value of 40% was used. This corresponds to the reported removal rates which range from 20 to 60% (6). If conditions warranted, nearly all fruits and vegetables could be processed by similar techniques in the home with about the same factors of reduction (13).

After the standardized reduction factors were used, it was necessary to develop some method of selecting maximal vitamin content and minimal ^{90}Sr contribution. This was done by ranking each fruit and vegetable according to a vitamin/^{90}Sr selection factor. These rankings determined the nature and direction of the transfers between different fruits and vegetables and were calculated in the following manner:

$$\text{Selection factor} = \frac{\text{nutritive content (vitamins A, C)}}{^{90}\text{Sr content (pCi } ^{90}\text{Sr/kg)}}$$

Thus, the higher the selection factor the more substitutable the food item insofar as vitamin content was concerned. Systems of linear equations were then used to quantify the actual interchangeability coefficients between specific fruits or vegetables. Solutions to the equations in terms of consumption changes could then be evaluated

for changes in ^{90}Sr intake. For example, the normal intake of cantaloupes, grapefruit and oranges would yield:

Vitamin A = 163,608 IU/year
Vitamin C = 10,716 mg/year
^{90}Sr = 57 pCi/year

Deleting oranges and substituting grapefruit or cantaloupes in the following manner could reduce the ^{90}Sr intake to 26 pCi/year:

C_g (vitamin A_g) + C_c (vitamin A_c) = 163,608
C_g (vitamin C_g) + C_c (vitamin C_c) = 10,716

Solving for C_g, the consumption of grapefruit = 24.52 kg/year and for C_c, the consumption of cantaloupes = 4.24 kg/year. This would compare with normal intakes of 8.88 and 11.19 kg/year for grapefruit and cantaloupes, respectively. Similar calculations with apricots and grapefruit indicate an interchangeability coefficient of 1.24/1.00 (apricots/grapefruit). These coefficients can be used to determine the direction of substitutions among products. Successive eliminations or substitutions of this type were used to obtain the minimal ^{90}Sr intake.

The relative rankings of selection factors over the 3-year period are shown in table 3 for vitamin A and ascorbic acid. The highest ranked items for vitamin A were apricots, cantaloupes and carrots; those for ascorbic acid were peppers, grapefruit and cauliflower.

RESULTS

Tables 4 and 5 present the results of calculations that show the effects on average annual intake of ^{90}Sr that could be brought about by: (a) processing of all items; (b) processing plus restricted substitution which emphasizes taste and preference selectivity, denoted as Substitution 1 (this substitution was made by establishing sub-classes of fruits and vegetables on the basis of compatability and use criteria such as for dessert, salad, cooking, and snacks); and (c) processing plus maximal substitution without concern for taste or preferred usage and complete elimination of many items, denoted as Substitution 2.

TABLE 3

Relative ranking of major fruits and vegetables by selection factors (vitamin/^{90}Sr ratio) [1]

Fruits			Vegetables		
	Vitamin A	Ascorbic acid		Vitamin A	Ascorbic acid
Apples	M	M	Leafy:		
Apricots	H	M	Broccoli	H	H
Cantaloupes	H	H	Cabbage	L	M
Cherries	M	H	Cauliflower	L	H
Cranberries	L	L	Celery	L	L
Grapefruit	M	H	Kale	M	M
Grapes	L	L	Lettuce	M	L
Nuts	L	L	Spinach	H	L
Oranges	M	H	Other:		
Peaches	H	M	Artichokes	L	M
Pears	L	L	Asparagus	H	H
Pineapple	M	H	Beets	L	L
Plums and prunes	H	L	Carrots	H	L
Strawberries	L	M	Cucumbers	M	L
Watermelon	H	M	Green beans	M	M
			Lima beans	M	H
			Other beans, dried	L	L
			Onions	L	M
			Peas, green	M	M
			Peppers	H	H
			Potatoes	L	H
			Pumpkin and squash	M	M
			Sweet potatoes	H	L
			Tomatoes	H	H

[1] Selection factors (vitamin/^{90}Sr ratio):
Vitamin A: high (H) = 30,000 to 2,000; medium (M) = 2,000 to 400; low (L) = 400 to 0.
Ascorbic acid: high (H) = 1,200 to 100; medium (M) = 100 to 15; low (L) = 15 to 0.

TABLE 4

Effect of various processing and substitution practices on ^{90}Sr intake in the United States, three-year average 1961–63

Fruits	^{90}Sr intake			
	Average	Average if all processed	Average under substitution [1]	
			1	2
	pCi/year	*pCi/year*	*pCi/year*	*pCi/year*
Apples	38	18	9	—
Apricots	1	1	3	5
Cantaloupe	11	7	7	8
Cherries	6	6	7	—
Cranberries	16	13	—	—
Grapefruit	9	6	12	20
Grapes	67	22	8	—
Nuts	59	59	—	—
Oranges	96	44	25	17
Peaches	42	26	13	—
Pears	13	12	6	—
Pineapple	4	3	3	—
Plums and prunes	2	2	5	3
Strawberries	22	16	18	—
Watermelon	22	14	7	—
Total fruits	408	249	123	53
Reduction, %		39	70	87

[1] Substitution 1 classifies fruits and vegetables into use, taste, and preference sub-categories so that dietary changes are in accord with normal practices. Substitution 2 uses maximal possible reductions without concern for taste or preference criteria.

TABLE 5

Effect of various processing and substitution practices on ^{90}Sr intake in the United States, three-year average 1961–63

Vegetables	^{90}Sr intake			
	Average	Average if all processed	Average under substitution [1]	
			1	2
	pCi/year	*pCi/year*	*pCi/year*	*pCi/year*
Leafy:				
Broccoli	3	3	9	34
Cabbage	64	30	12	35
Cauliflower	5	3	2	—
Celery	42	25	8	—
Kale	3	3	10	—
Lettuce	136	73	24	—
Spinach	38	38	13	—
Other:				
Artichokes	1	1	—	—
Asparagus	2	1	7	—
Beets	10	8	4	—
Carrots	35	30	46	56
Cucumbers	15	12	4	—
Green beans	39	24	8	—
Lima beans	2	2	1	—
Other beans, dried	14	14	4	—
Onions	40	25	10	—
Peas, green	19	16	19	—
Peppers	2	1	4	2
Potatoes	216	94	97	94
Pumpkin and squash	1	1	1	—
Sweet potatoes	66	46	16	—
Tomatoes	36	35	37	21
Total vegetables	789	485	336	242
Reduction, %		38	57	69

[1] Substitution 1 classifies fruits an vegetables into use, taste, and preference sub-categories so that dietary changes are in accord with normal practices. Substitution 2 uses maximal possible reductions without concern for taste or preference criteria.

Data in tables 4 and 5 show that processing alone could cause a reduction of about 40% in ^{90}Sr intake from fruits and vegetables; restricted substitution in addition to complete processing, a reduction of 70 to 90%.

Table 6 presents a summary of the overall effects of the modifications proposed. It is noted that the initial losses in total bulk from a farm weight basis to an all-processed form averaged about 19%, whereas losses under Substitutions 1 and 2 averaged 29 and 44%, respectively. Total intake of both vitamin A and ascorbic acid remained at the same relative levels for all modifications. Calcium intake was reduced by 17% during the processing and preparation activities. Substitutions 1 and 2 brought about reductions in calcium intake of 37 and 44%, respectively. In terms of total diet the calcium reductions are considered insignificant. However, the reductions in ^{90}Sr intake from 1197 pCi/year to 734 by processing and to 459 and 295 pCi/year through Substitution 1 and 2 are relatively larger, representing a maximal 75% reduction in ^{90}Sr intake from fruits and vegetables.

Similarly the ^{90}Sr/g Ca ratio was reduced from 27.7 to 19.8, 16.9 and 12.2 pCi by the mechanism of processing and the two substitution exchanges.

DISCUSSION

Since fruits and vegetables are only one of the major food categories it is necessary to examine the effect of these changes on the total diet. Table 7 shows the results of such changes for a diet representing the New York City area. The major diet categories are shown with their ^{90}Sr and calcium contributions. The category for fruits and vegetables has been listed separately to show the differences in intake arising from processing and substitution practices. Total diet summaries are presented under each of the conditions considered for the fruit and vegetable category (Substitution 1 considering the taste and preference criteria and Substitution 2 considering the maximal ^{90}Sr reduction). Reductions in total dietary ^{90}Sr intake of 13% are shown for processing fruits and vegetables, whereas processing coupled with substitution alternatives reduce the total intake by 21 and 26%. Similar reductions occur in the

TABLE 6

Effects of various ^{90}Sr reduction practices on consumption, calcium and ^{90}Sr levels in the United States, three-year average 1961–63

	Intake levels			
	Unprocessed (farm weight)	All processed [1] (edible equivalent)	Substitution [2]	
			1	2
Fruit:				
Total bulk, kg	78.1	62.5	54.4	44.1
Total calcium, g	13.6	11.9	9.5	8.2
Total ^{90}Sr, pCi	408	249	123	53
^{90}Sr/Ca, pCi/g	30.0	20.9	12.9	6.5
Vegetables:				
Total bulk, kg	134.7	109.8	96.4	75.7
Total calcium, g	29.6	25.1	17.6	15.9
Total ^{90}Sr, pCi	789	485	336	242
^{90}Sr/Ca, pCi/g	26.6	19.3	19.1	15.2
Total fruits and vegetables:				
Bulk, kg	212.8	172.3	150.8	119.8
Calcium, g	43.2	37.0	27.1	24.1
^{90}Sr, pCi	1,197	734	459	295
^{90}Sr/Ca, pCi/g	27.7	19.8	16.9	12.2

[1] Processed in this context refers to the standard preparation practices commonly available to homemakers (as washing and peeling).

[2] Substitution 1 classifies fruits and vegetables into use, taste and preference sub-categories so that dietary changes are in accord with normal practices. Substitution 2 uses maximal possible reductions without concern for taste or preference criteria.

TABLE 7

Relationship of substitution practices among fruits and vegetables to total annual dietary ^{90}Sr intake, example for New York City area, three-year average 1961–63.

	^{90}Sr intake [1]	Calcium intake	pCi ^{90}Sr/g Ca
	pCi/year	*g/year*	
Dairy products	3762	238.22	15.9
Grain products	1039	58.72	17.8
Meat, fish and eggs	162	44.78	3.4
Diet subtotal	4963	341.72	14.5
Fruits and vegetables (F and V):			
Unprocessed	2582	64.07	40.3
Processed	1583	53.18	29.8
Substitution 1 [2]	989	38.82	25.5
Substitution 2 [2]	636	34.63	18.4
Total diet with:			
F and V unprocessed	7545	405.79	19.1
F and V processed	6546	394.90	16.6
F and V Substitution 1	5952	380.54	15.1
F and V Substitution 2	5599	376.35	14.2

[1] ^{90}Sr determinations obtained from Tri-City Diet Study, Health and Safety Laboratory Fallout Program Reports HASL-115, 1961 through HASL-144, 1964. U.S. Atomic Energy Commission New York Operations, New York.

[2] Substitution 1 classifies fruits and vegetables into use, taste and preference sub-categories so that dietary changes are in accord with normal practices. Substitution 2 uses maximal possible reductions without concern for taste or preference criteria.

^{90}Sr/g Ca level. Calcium intake under all conditions has been maintained at 93 to 97% of initial levels. In addition, vitamin A, ascorbic acid and iron intake levels were also maintained well above recommended standards.

The effect of substituting under a non-dairy diet would produce some additional reductions in the ^{90}Sr intake. However, the deletion of dairy products would result in a major calcium loss, a loss which could not be recovered by increases in the consumption of other foods. An example of this approach is shown in table 8 where dairy products have been deleted from the diet representing the New York City area. Although the net ^{90}Sr intake has been reduced by 46 to 67%, the calcium intake has also been reduced by more than 60%. Such a diet would require considerable calcium supplementation to maintain adequate nutrition (intake of 138–156 g calcium, whereas recommended diet levels are 290–510 g depending on the age of the subject). Supplemental additions of inorganic calcium could be used to further reduce the ^{90}Sr/g Ca level but such practices must await the proper medical investigations before being available for general usage.

TABLE 8

Example of substitution practices among fruits and vegetables for a non-dairy diet, New York city area, three-year average 1961–63

	^{90}Sr intake [1]	Calcium intake	pCi ^{90}Sr/g Ca
	pCi/year	*g/year*	
Grain products	1039	58.72	17.8
Meat, fish and eggs	162	44.78	3.4
Diet subtotal	1201	103.50	11.6
Fruits and vegetables (F and V):			
Unprocessed	2582	64.07	40.3
Processed	1583	53.18	29.8
Substitution 1 [2]	989	38.82	25.5
Substitution 2 [2]	636	34.63	18.4
Total diet with:			
F and V unprocessed	3783	167.57	22.6
F and V Processed	2784	156.68	17.8
F and V Substitution 1	2190	142.32	15.4
F and V Substitution 2	1837	138.13	13.3

[1] ^{90}Sr determinations obtained from Tri-City Diet Study, Health and Safety Laboratory Fallout Program Reports HASL-115, 1961 through HASL-144, 1964. U.S. Atomic Energy Commission New York Operations, New York.

[2] Substitution 1 classifies fruits and vegetables into use, taste and preference sub-categories so that dietary changes are in accord with normal practices. Substitution 2 uses maximal possible reductions without concern for taste or preference criteria.

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Evidence that Creatine May Be One Factor in the Low Transamidinase Activities of Kidneys from Protein-depleted Rats [1]

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ABSTRACT Experiments were carried out in an attempt to determine whether high tissue levels of creatine are a factor in the low transamidinase activities of kidneys from rats fed a protein-free diet. Rats were killed at varying lengths of time after having been fed a protein-free diet and 7 days after having been fed a complete diet supplemented with creatine. Kidney transamidinase activities, in vitro, and the amounts of creatine in the blood and kidneys of the rats were determined. Two separate and distinct phases in the loss of kidney transamidinase activity were observed in the rats fed the protein-free diet. These phases were separated by a period of time during which the enzyme values remained constant. The amounts of creatine in the blood and kidneys were normal in the first phase but in the second phase were similar to those observed in rats fed complete diets supplemented with sufficient creatine to result in low kidney transamidinase activities. Elevated tissue levels of creatine were probably not a factor in the first phase but are suggested as a possible factor in the second phase.

A large number of enzyme activities have been measured in tissues from animals fed diets deficient in protein or certain essential amino acids. Tissues from animals fed the deficient diets usually have lower enzyme activities, in vitro, than tissues from animals fed complete diets. The mechanisms of the alterations of the enzyme activities have for the most part remained obscure. Recent observations about rat kidney transamidinase have made it possible to investigate a possible reason for its alteration in protein depletion.

Kidneys from rats fed a protein-free diet have only a fraction of the transamidinase activities, in vitro, as kidneys from rats fed a complete diet (1–6). It appeared possible that elevated creatine levels may be a factor responsible for the low activities of kidneys from protein-depleted rats. Creatinuria has been observed in rats fed a protein-free diet (7). Kidneys from rats fed complete diets supplemented with creatine have much lower transamidinase activities than kidneys from rats fed the unsupplemented diets (4–6, 8–11).

Rats were killed at varying lengths of time after having been fed a protein-free diet. Also, rats were killed 7 days after having been fed a complete diet supplemented with varying amounts of creatine. Kidney transamidinase activities, in vitro, and blood and kidney levels of creatine were determined.

METHODS

Male rats of the Holtzman strain, 100 g, were housed individually and fed a complete purified diet that was a modification of a diet published previously (12). The diet contained: (in grams),[2,3] casein, 20; cornstarch, 53.7; corn oil, 20; salt mixture USP XIV, 4; DL-methionine, 0.25; and vitamin mixture, 1 or 2 g. Vitamin mixture A was used at the level of 2 g/100 g diet; and vitamin mixture B at the level of 1 g/100 g

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[1] These studies were supported in part by Public Health Service Research Grant no. A-2731 from the National Institute of Arthritis and Metabolic Diseases.
[2] Casein, salt mixture USP XIV, DL-methionine, and vitamin mixture A were obtained from Nutritional Biochemicals Corporation, Cleveland. The vitamin mixture was triturated in dextrose, and 1 kg of the vitamin mixture contained the following: (in grams) vitamin A conc (200,000 units/g), 4.5; vitamin D conc (400,000 units/g), 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; Ca pantothenate, 3.0; (in milligrams) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35.
[3] Casein, salt mixture USP XIV, DL-methionine, and vitamin mixture B were obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. The vitamin mixture was triturated in cornstarch and 0.454 kg of the vitamin mixture contained the same amounts of vitamins as in 1 kg of vitamin mixture A.

diet. The protein-free diet was identical with the complete diet with the exception that the casein was replaced with an equal weight of cornstarch. All rats had access to the complete diet and water, ad libitum, for 2 weeks prior to being fed the experimental diets so that they would have uniformly high levels of transamidinase activity in their kidneys. The rats had access to the experimental diets, ad libitum. The rats were decapitated and the blood was collected in beakers containing potassium oxalate. The kidneys were saved for the analysis of creatine and transamidinase. A 5% homogenate of the kidneys was made in distilled water with a Potter-Elvehjem homogenizer; 0.5-ml aliquots of the homogenate were used for transamidinase activities, in vitro. The procedure was as published previously (1) with the modification that the glycine and arginine were dissolved in 0.099 M phosphate buffer, pH 7.4. The total nitrogen was determined by a micro-Kjeldahl procedure (13), and the transamidinase activities were expressed as micrograms of guanidinoacetic acid formed per milligram of kidney nitrogen per hour. Protein-free filtrates of the kidney homogenates were made by adding to 3 ml of 5% kidney homogenate 5 ml of H_2O, 3 ml of 10% sodium tungstate and 3 ml of 2/3 N H_2SO_4. The protein-free filtrates of the blood (7) and kidney were neutralized to pH 5–7 and used for the determination of creatine (14).

Each dot on all figures represents an individual value. In figures 1–6 the lines were drawn through the averages of the individual values.

RESULTS AND DISCUSSION

The effects of the length of time of the feeding of a protein-free diet on kidney transamidinase activities, in vitro, and blood and kidney creatine levels were determined. A group of 40 male rats of the Holtzman strain, weighing approximately 100 g, was fed a complete purified diet for 2 weeks. The rats, then weighing approximately 170 g, were then fed the protein-free diet. A group of 5 to 6 rats was killed each day beginning on the last day of the complete dietary regimen and ending on the sixth day after initiating the protein-free diet. The transamidinase activities of kidneys from these rats are shown in figure 1. The line was drawn through the means of the daily transamidinase activities. There was a decline of transamidinase activities with time fed the protein-free diet except for the period between days 3 and 4. Three similar additional experiments have been conducted and the patterns of the graphs were nearly identical to the pattern in figure 1 in that the decline of enzyme activities on days 1 to 3 and 5 to 6 was separated by a period of time (days 3 to 4) in which the activities remained relatively constant. The slopes (± 1 SE) of the fitted least-squares lines for each of the 3 phases in transamidinase activities for all 4 experiments are listed in table 1. A combined one-sided *P*-value (15) for the null hypothesis that each regression curve is a level line between days 3 and 4 against the alternative hypothesis that it is a declining line is 0.44; thus it would not be rejected by a test of hypothesis.

The amounts of creatine present in the kidneys from the rats fed the protein-free

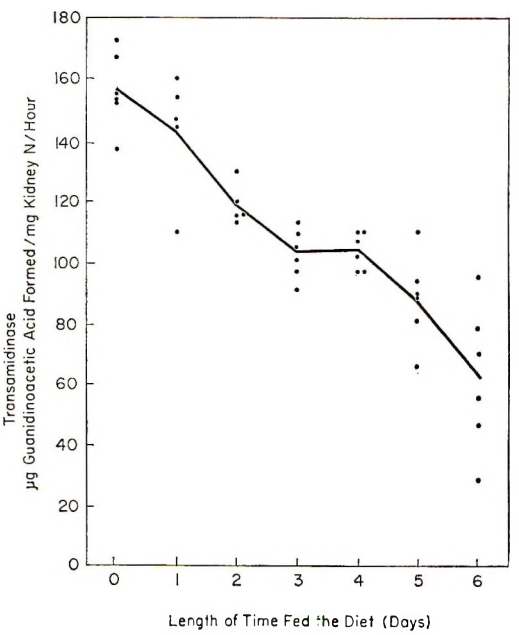

Fig. 1 Transamidinase activities of kidneys from rats fed a protein-free diet for varying lengths of time.

TABLE 1

Slopes of the fitted least squares lines for kidney transamidase activities of rats fed a protein-free diet for varying lengths of time

Exp. no.	Days fed protein-free diet					
	1 to 3	df	3 to 4	df	5 to 6	df
1 (fig. 1)	−0.190 ± 0.071 [1]	14	0.007 ± 0.43	10	−0.253 ± 0.016	10
2	−0.078 ± 0.015	12	−0.018 ± 0.027	10	−0.118 ± 0.035	7
3	−0.062 ± 0.019	7	−0.017 ± 0.040	4	−0.043 ± 0.097	2
4	−0.070 ± 0.022	7	0.000 ± 0.023	4	−0.107 ± 0.047	4

[1] Slopes ± 1 SE.

diet are shown in figure 2. Kidneys from rats fed the complete diet for 2 weeks and from rats fed the protein-free diet for 1 to 4 days contained similar amounts of creatine — approximately 2.5 μg/mg kidney nitrogen. Kidneys from rats fed the protein-free diet for 5 to 6 days had much greater amounts of creatine (6 and 10 μg/mg kidney nitrogen, respectively) than the rats fed the diet for shorter periods of time.

The amounts of creatine observed in the blood of the rats fed the protein-free diet are shown in figure 3. Blood from rats fed a complete purified diet for 2 weeks and rats fed a protein-free diet for 1 to 4 days

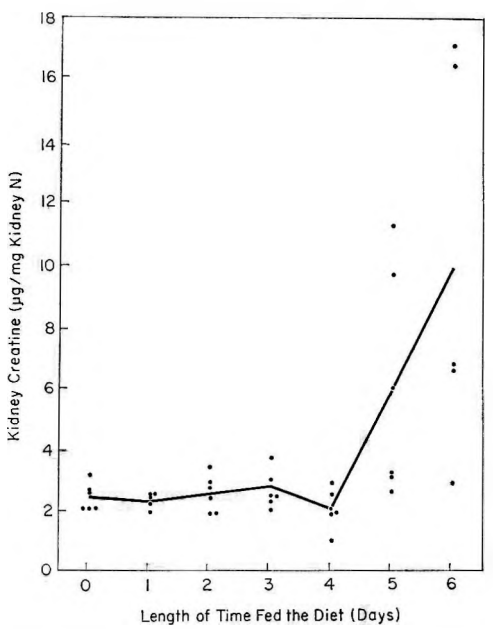

Fig. 2 Creatine in kidneys from rats fed a protein-free diet for varying lengths of time.

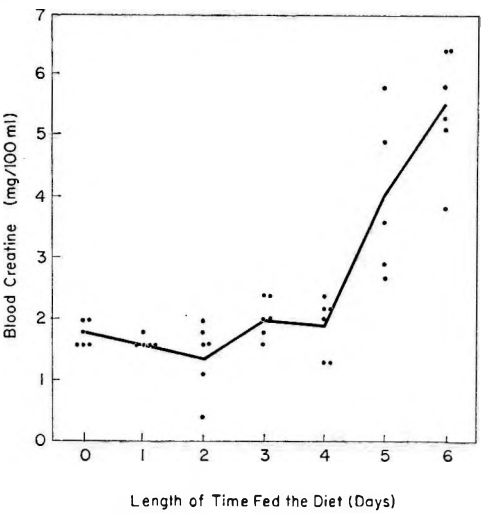

Fig. 3 Creatine in the bloods from the rats fed the protein-free diet for varying lengths of time.

had similar amounts of creatine — approximately 2 mg/100 ml. Blood from rats fed a protein-free diet for 5 to 6 days contained larger amounts of creatine (4 and 5 mg/100 ml, respectively) than rats fed the diet for shorter periods of time.

The effects of feeding a complete purified diet supplemented with varying amounts of creatine upon kidney transamidinase activities and blood and kidney creatine levels were determined. A group of 70 male rats of the Holtzman strain was fed a complete purified diet for 2 weeks. The rats, then weighing approximately 170 g, were fed the same diet supplemented with varying amounts of creatine (0.08 to 0.18 g/100 g diet). Each diet was fed to a group of 10 rats and the rats were killed after having been fed the

creatine-supplemented diets for 7 days. It had been determined in previous experiments that kidneys from rats fed a creatine-supplemented diet had a gradual decline in transamidinase activities with length of time fed the diet for as long as 5 to 6 days. The activities of kidneys from rats fed creatine-supplemented diets for longer periods of time remained at a constant low level.

The transamidinase activities of kidneys from the rats fed the creatine-supplemented diets are shown in figure 4. It is considered that rats fed the complete diet supplemented with 0.10 g or more of creatine/100 g diet had slightly lower ($P < 0.001$) enzyme activities than the rats fed the unsupplemented diet.

In figure 5 are plotted the amounts of creatine in the same kidneys from which the transamidinase activities were plotted in figure 4. The average amount of creatine present in the rats fed the 0.12% creatine-supplemented diet was approximately 5 μg/mg kidney nitrogen. Although the average amount of creatine in the kidneys from the rats fed the 0.12% diet was twice that in kidneys from rats fed the diets supplemented with smaller amounts of creatine, this was not a significantly larger amount because of the large standard deviations of the values.

The amounts of creatine in the blood from the rats fed the creatine-supplemented diets are plotted in figure 6. The blood from the rats fed the 0.12% creatine-supplemented diet had a significantly greater ($P < 0.001$) average amount of creatine (3.5 mg/100 ml) than blood from rats fed the unsupplemented diets (1.5 mg/100 ml).

The data plotted in figures 1 to 6 indicate a considerable variation in kidney transamidinase activities and also in tissue levels of creatine — particularly in the later stages of protein-depletion and with the larger amounts of creatine in the complete diet. In figures 7 and 8 are plotted the individual blood and kidney levels of creatine against the kidney transamidinase activities noted for the rats fed the protein-free diet. The decline of enzyme activities without any increase in blood and kidney creatine levels which occurred on days 1 to 3 is apparent. The decline of enzyme activities along with the increase

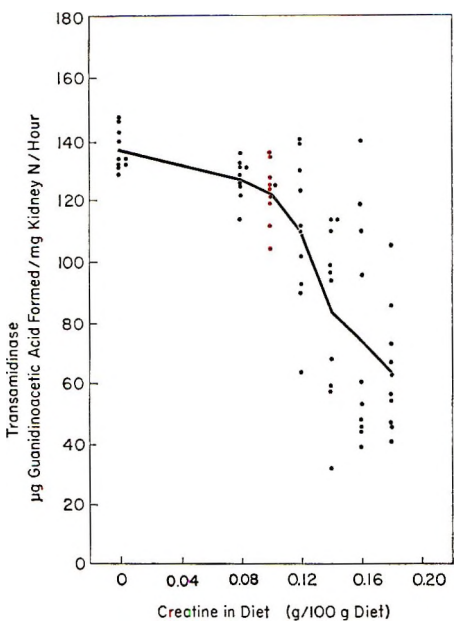

Fig. 4 Transamidinase activities of kidneys from rats fed a complete diet, supplemented with varying amounts of creatine, for 7 days.

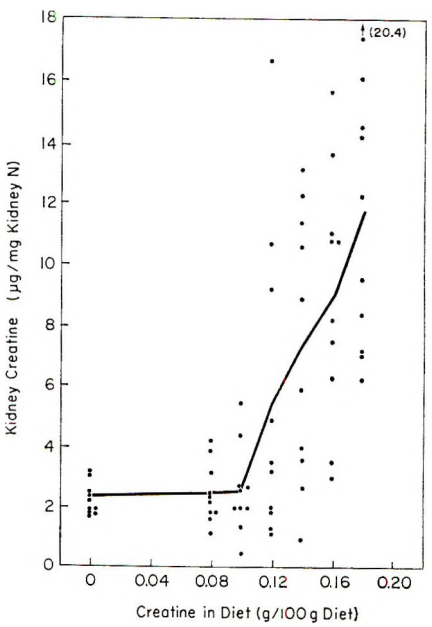

Fig. 5 Creatine in the kidneys from rats fed the complete diet supplemented with varying amounts of creatine.

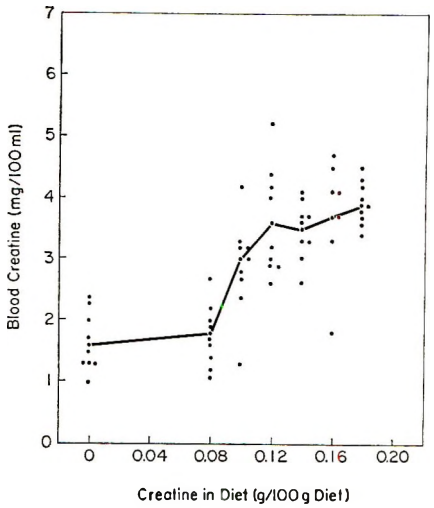

Fig. 6 Creatine in bloods from rats fed the the complete diet supplemented with varying amounts of creatine.

in blood and kidney creatine levels (which occurred on days 5 to 6) is also apparent. In figures 9 and 10 are plotted the data from the rats fed the creatine-supplemented diets. There was a good correlation between high levels of creatine in the blood and kidney and low kidney transamidinase activities.

It is believed that there were two separate and distinct phases in the loss of transamidinase activity in protein depletion. There were two time periods (days 1 to 3 and 5 to 6) in which a decline of enzyme activity was observed. These periods of decline of enzyme activity were separated by a period in which the enzyme activities remained constant (days 3 to 4). There was no significant increase in the amounts of creatine present in the blood and kidneys from the rats fed the protein-free diet during the first phase (days 1 to 3). It was therefore concluded that creatine probably was not a factor responsible for the decline of activities during this time period. It is not possible to state the reason or factor responsible for the first phase in the loss of enzyme activity.

Creatine may well be a factor responsible for the decline of enzyme activities in the second phase (days 5 to 6). Kidneys from rats killed after having been fed the protein-free diet for 5 to 6 days had significantly greater amounts of creatine in their blood and kidneys than observed in the rats killed after 1 to 4 days. Furthermore, the average amount of creatine observed on day 5 (6 μg/mg kidney nitrogen and 4 mg/100 ml blood) was similar to the average amount present in the blood and kidneys from rats fed a complete diet supplemented with 0.12% creatine (5 μg/mg kidney nitrogen and 3.5 mg/100 ml blood). The kidneys from the rats fed the

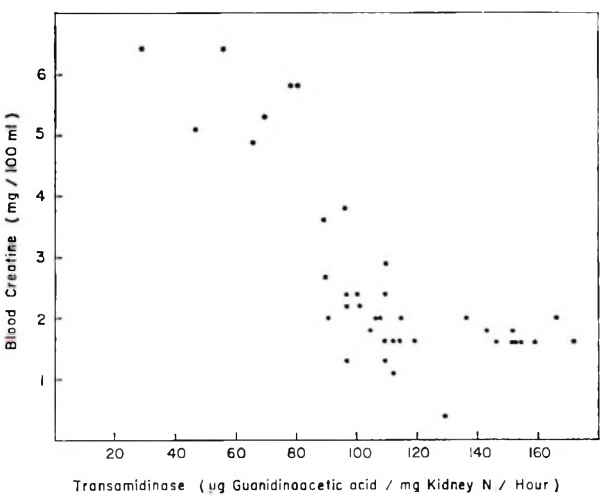

Fig. 7 Kidney transamidinase activities and amounts of creatine found in bloods from rats fed the protein-free diet.

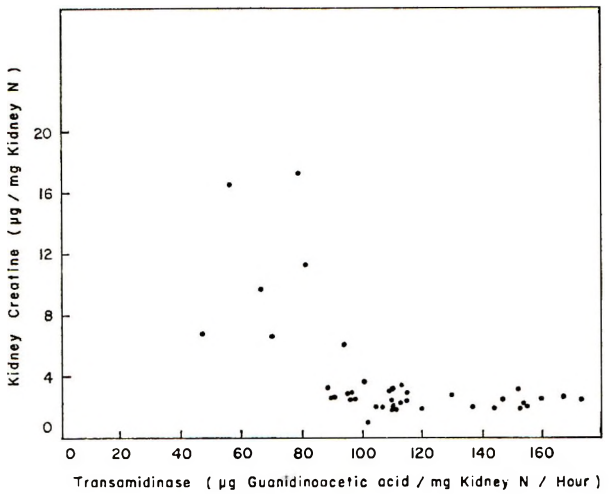

Fig. 8 Kidney transamidinase activities and amounts of creatine found in kidneys from rats fed the protein-free diet.

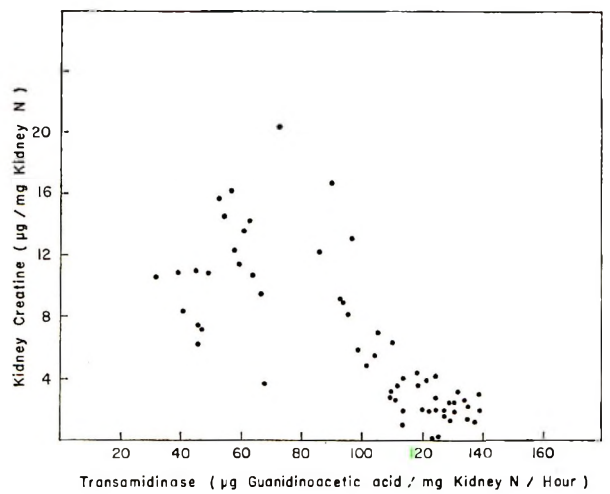

Fig. 9 Kidney transamidinase activities and amounts of creatine found in kidneys from rats fed the creatine-supplemented diets.

0.12% creatine diet had significantly lower enzyme activities than the kidneys from rats fed the unsupplemented diet.

It is not known why rats fed a creatine-supplemented diet have low kidney transamidinase activities, in vitro. It has been reported by Walker (6) that creatine, added to kidney homogenate, has no effect on transamidinase activities, in vitro. This report has been confirmed in our laboratories. Also it has been determined in our laboratories that homogenates of kidney from protein-depleted rats do not inhibit normal rat kidney transamidinase activities. Therefore, it is not possible to state that the large amount of creatine in the blood and kidneys of rats fed the creatine-supplemented diets was a direct factor or cause of the low enzyme activities. At present it can be stated only that there was a good correlation between large amounts of creatine in the blood and kidneys and the low enzyme activities in both the protein-depleted rats (days 5 to 6) and

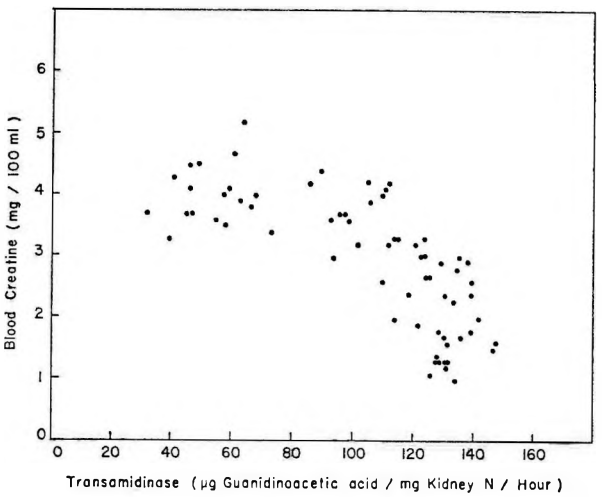

Fig. 10 Kidney transamidinase activities and amounts of creatine found in blood from rats fed the creatine-supplemented diet.

the rats fed a complete diet supplemented with creatine. It is true that the appearance of the large amount of creatine on days 5 to 6 of protein depletion may be coincidental with the depression of enzyme activities. The fact that the 2 phases of depression of enzyme activities were always separated by a period (days 3 to 4) in which enzyme activities remained constant is interpreted to indicate that two separate and distinct factors or causes were involved in the loss of transamidinase in protein-depletion. There certainly was no indication that creatine was a factor in the decline of enzyme activities during the first 3 to 4 days that the rats were fed the protein-free diet.

A circadian periodic variation in kidney transamidinase activities, in vitro, has been reported (16). Mice, standardized for one week prior to sampling, in light from 0600 to 1800 alternating with darkness, have been found to have a variation in activities with clock hour. These variations were small — the differences between the maximal and minimal activities were approximately 15% of the mean values. Not only does transamidinase have what is considered to be a low amplitude rhythm, but this rhythm can be demonstrated only after a period of light-dark standardization. The times of killing the animals in the present study were not recorded. However, it can be stated that to the best of our knowledge all animals were probably killed between 0800 and 1200. Furthermore, the animals had not been standardized to a light-dark regimen at any time. It is believed that the alterations in the transamidinase activities observed in the present report were independent of any possible circadian periodic variation.

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Carcass Transamidinase Activities, in vitro, and Rates of Creatine Synthesis, in vivo, in Normal and Protein-depleted Rats [1]

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ABSTRACT Experiments were conducted in an attempt to determine whether there was any correlation between carcass transamidinase activities, in vitro, and rates of creatine synthesis, in vivo. Protein depletion was the method used to produce alterations in the transamidinase activities. Rats were fed either complete or protein-free diets for a period of 7 days and then given intraperitoneal injections of solutions of glucose, or arginine plus glycine. Carcass transamidinase activities, in vitro, and the amounts of creatine and creatinine in the urine and carcasses were determined. The difference in the amounts of creatine plus creatinine between the rats injected with arginine-glycine and the glucose-injected rats was defined as the rate of creatine synthesis, in vivo. There was a good correlation between rates of creatine synthesis, in vivo, and carcass transamidinase activities, in vitro, that is, both measurements were 2 times as great in the rats fed the complete diet as in the rats fed the protein-free diet.

Transamidinase activities, in vitro, have been observed in rat kidney, pancreas, heart, muscle, spleen, testes, brain, thymus, lung, and decidua (1–6). Kidney, pancreas and decidua have greater activities than the other above listed tissues. However, the sum of the calculated total tissue activities of spleen, muscle, lung, brain and testes was similar to the sum of the calculated total tissue activities of kidney and pancreas (6). Much has been reported about the alteration of kidney transamidinase activities, in vitro, by various experimental procedures, for example, protein-depletion and creatine supplementation (2, 6, 7–13). There has been some indication that there may be little correlation between kidney transamidinase activities, in vitro, and rates of creatine synthesis, in vivo (14, 15).

In the present series of experiments attempts were made to determine whether there was any correlation between the rates of synthesis of creatine, in vivo, and the carcass transamidinase activities, in vitro. Normal and protein-depleted rats were given intraperitoneal injections of either a solution of arginine plus glycine or a solution of glucose. After a period of time (2 or 4 days), during which urine was collected, the animals were killed and homogenates of their carcasses prepared. The transamidinase activities, in vitro, of the carcasses and the amounts of creatine plus creatinine in the urine and carcasses were determined. It was found that the amount of creatine plus creatinine in the carcass plus urine of the rats that had received injections of arginine and glycine was greater than in the rats that had received injections of glucose. The differences in the amounts of creatine plus creatinine (expressed as mg creatinine/100 g carcass) between the rats injected with arginine-glycine and the glucose-injected rats were defined as the rate of creatine synthesis, in vivo.

METHODS

Male albino rats of the Holtzman strain weighing approximately 100 g, were fed either a complete (16) or protein-free diet for 7 days. The protein-free diet was identical with the complete diet except that the casein was replaced with an equal amount of cornstarch. The rats were

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housed in metabolism cages with access to food and water ad libitum. L-Arginine (free-base) and glycine were dissolved in distilled water and adjusted to pH 7.4 by allowing CO_2 gas to bubble through the solution. Creatine or glucose were dissolved in distilled water. The rats were injected with the test solutions and were continued with the same diets as fed the first 7 days. The urine collections were started immediately following the injections of the test solutions and were continued for 2 to 4 days. The urine collection flasks contained toluene and a small amount of thymol. The rats were killed by intraperitoneal injections of 0.5 ml/100 g body weight of a solution of 50 mg/ml of pentobarbital sodium.[2] The skins, claws, and tails were removed and discarded. A 10% homogenate of the carcass (including viscera), in ice cold water, was prepared with a 3.8-liter Waring Blendor. The transamidinase activities, in vitro, were determined by incubating 2-ml aliquots of the carcass homogenates with 0.5 ml of a solution containing 25 μmoles each of canavanine sulfate and glycine in 0.33 M phosphate buffer, pH 7.4 (6). One-milliliter aliquots of the incubation mixture were withdrawn prior to and after a 7-hour incubation period at 37° with shaking and with an air phase over the vessels. The protein in these aliquots was precipitated by the addition of 3 ml of 0.3 N $Ba(OH)_2$ followed by 3 ml of 5% $ZnSO_4$ (17). Two milliliters of the protein-free filtrate were used for the modified Sakaguchi color reaction (17). The guanidinoacetic acid formed was directly proportional to the time of incubation (fig. 1) and to the amount of carcass homogenate (fig. 2). The transamidinase activities were expressed as milligrams guanidinoacetic acid formed per gram (wet weight) of carcass per 7 hours.

To 10 ml of the carcass homogenate were added 170 ml of H_2O, 10 ml of 2/3 N H_2SO_4 and 10 ml of 10% sodium tungstate. The protein-free filtrate was adjusted to pH 5–7 and analyzed for creatine and creatinine (17). To 10 ml of the urine were added 5 ml of 10% sodium tungstate, 5 ml of 2/3 N H_2SO_4 and 80 ml of H_2O. The protein-free filtrate was adjusted to pH 5–7 and analyzed for crea-

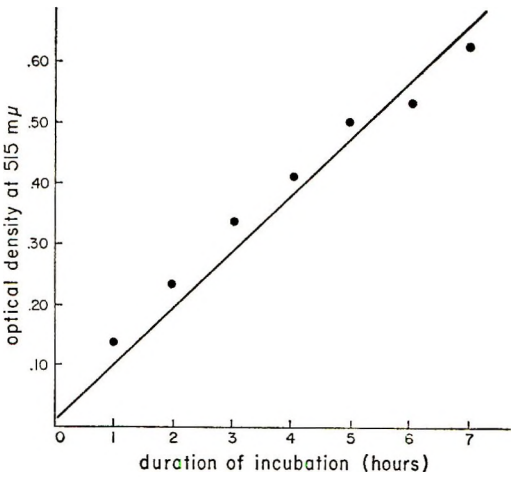

Fig. 1 Effect of duration of incubation on guanidinoacetic acid formation by normal rat carcass homogenate. Eight milliliters of 10% rat carcass homogenate were mixed with 2.0 ml of a solution containing 125 μmoles each of canavanine sulfate and glycine in 0.33 M phosphate buffer, pH 7.4. One-milliliter aliquots were removed prior to and after 1-hour incubation periods for guanidinoacetic acid analysis as under Methods. The total incubation period was 7 hours.

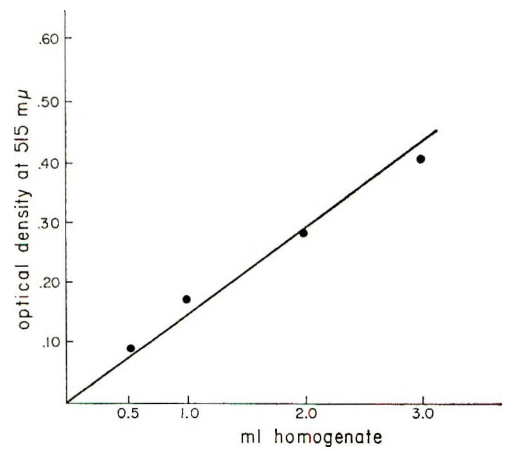

Fig. 2 Effect of amount of rat carcass homogenate on guanidinoacetic acid formation. Flasks containing 0.5, 1.0, 2.0, or 3.0 ml of rat carcass homogenate were mixed with 0.5 ml of a solution containing 25 μmoles each of canavanine sulfate and glycine in 0.33 M phosphate buffer pH 7.4. The total volume in each flask was made up to 3.5 ml with water. Incubation was for 7 hours and guanidinoacetic acid analysis was as described under Methods.

[2] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.

tine and creatinine (17). The creatine and the creatinine were both expressed as milligrams of creatinine/100 g carcass or as milligrams of creatinine/100 g carcass excreted/48 hours, or as milligrams of creatinine/100 g carcass excreted/96 hours. The term carcass was defined as the animal without skin, claws and tail. The viscera were included in the carcass homogenate. All data are listed in table 1 as the average values ± the standard deviation.

RESULTS AND DISCUSSION

A. *Fate of small doses of creatine injected intraperitoneally into normal rats.* It has been reported previously that 89% of a large dose (227 mg) of creatine injected into rats was recovered as creatine plus creatinine (18). A group of 20 male albino Holtzman rats, weighing approximately 100 g, were fed a creatine-free, complete purified diet (16) for 7 days. The rats were housed two to a cage with access to food and water ad libitum. On the seventh day 10 rats were given intraperitoneal injections of 10 ml of an aqueous solution of creatine, 2.6 mg/ml (equivalent to 2.24 mg creatinine/ml). The remaining 10 rats were given intraperitoneal injections of 10 ml of an aqueous solution of glucose, 2.6 mg/ml. All rats were killed 2 days later. The procedures for killing the rats and for the analysis of carcass and urine creatine and creatinine were as described in Methods.

The results are shown in table 1 (series 1). The average of the total carcass and urine creatine plus creatinine of the rats that were injected with glucose was 187 mg creatinine/100 g carcass, and the value for the rats that were injected with creatine was 210 mg creatinine/100 g carcass. The average carcass weight of both groups was 115 g; therefore the amount of creatine (as creatinine) recovered was 23×1.15 or 26.4 mg.

Statistically, the question arises: does the quantity 26.4, which is the difference of the averages of groups 1 and 2, depart significantly from the target value of 22.4, the number of milligrams of creatine (as creatinine) injected? The usual calculation (18) from the individual values yields a standard error for the sample difference of 6.9. Hence the departure of the recovered from the injected, 26.4 to 22.4, relative to the standard error is 0.43. The probability of a normal deviate of this absolute magnitude or greater arising by chance is 67%. Hence the values 22.4 and 26.4 do not differ significantly. Moreover, in view of the large significance probability, the quantities 22.4 and 26.4 are statistically of the same order of magnitude (19).

It was believed, therefore, if the animal did synthesize relatively small amounts of creatine from the exogenous arginine and glycine, that the creatine would not be destroyed and furthermore could be detected by our techniques.

B. *Rate of creatine synthesis in rats injected with arginine + glycine.* Forty male albino Holtzman rats weighing approximately 100 g were divided into 2 groups of 20. One group was fed the complete diet and the other the protein-free diet. After the rats had been fed the diets for 7 days, rats from each group were given an intraperitoneal injection of arginine plus glycine or were given an intraperitoneal injection of glucose. The solutions contained 50 mg glucose/ml or 40.0 mg L-arginine (free base) and 17.5 mg glycine/ml. Both solutions were injected in amounts equivalent to 0.06 ml/g body weight. The animals were killed 2 days after the injection and the results are shown in table 1 (series 2 and 3). The total carcass and urine creatinine and creatine in the rats fed the complete diets and that received injections of glucose was 180 mg/100 g carcass. The value for the rats fed the complete diet and that received an injection of arginine plus glycine was 203 mg/100 g carcass. The difference between these 2 values, 23 mg creatinine, was defined as the rate of creatine synthesis in the rats fed the complete diet. The rate of creatine synthesis in the rats fed the protein-free diet was 244 − 234 or 10 mg creatinine/100 g carcass. Thus the estimate of the rate of creatine synthesis, in vivo, in the protein-depleted rats was 10/23 or 43.5% of that in the normal rats.

The average transamidinase activities of the carcasses from the rats fed the normal diet (series 2, group 1 and group 2 in table 1)

TABLE 1

Amounts of creatine and creatinine in carcasses and urine, and carcass transaminase activities of rats fed a complete or protein-free diet and given intraperitoneal injections of either glucose or arginine + glycine

	Diet	Injections	No. of rats	Carcass creatinine and creatine	Urinary creatinine	Urinary creatine	Total carcass + urinary creatinine and creatine	Transamidinase activity as guanidinoacetic acid
				mg creatinine/ 100 g carcass	*mg creatinine/100 g carcass/48 hr*		*mg creatinine/ 100 g carcass*	*mg/g carcass/7 hr*
Series 1								
Group 1	complete	glucose	10	179 ± 14.8 [1]	7 ± 0.4	1 ± 0	187 ± 14.8	
Group 2	complete	creatine	10	197 ± 12.8	7 ± 0.5	6 ± 3.1	210 ± 14.6	
Difference				18	0	5	23	
Series 2								
Group 1	complete	glucose	10	172 ± 7.9	7 ± 0	1 ± 0	180 ± 7.8	0.731 ± 0.081
Group 2	complete	arginine, glycine	10	195 ± 6.8	6 ± 0.5	1 ± 0.4	203 ± 6.9	0.758 ± 0.120
Difference				23	0	0	23	
Series 3								
Group 1	protein-free	glucose	10	213 ± 7.7	7 ± 0.4	14 ± 3.7	234 ± 8.1	0.339 ± 0.042
Group 2	protein-free	arginine, glycine	10	222 ± 8.0	7 ± 0.5	14 ± 1.4	244 ± 8.0	0.386 ± 0.045
Difference				9	0	0	10	
					mg creatinine/100 g carcass/96 hr			
Series 4								
Group 1	complete	glucose	6	205 ± 6.0	14 ± 0	0 ± 0	219 ± 6.0	0.49 ± 0.02
Group 2	complete	arginine, glycine	5	225 ± 2.4	14 ± 0	20 ± 0	259 ± 2.4	0.48 ± 0.04
Difference				20	0	20	40	
Group 3	protein-free	glucose	7	227 ± 5.9	14 ± 0	9 ± 0	250 ± 5.9	0.21 ± 0.02
Group 4	protein-free	arginine, glycine	6	234 ± 4.0	14 ± 0	18 ± 0	266 ± 4.0	0.24 ± 0.02
Difference				7	0	9	16	

[1] All values are expressed as the average values ± 1 SD.

was estimated to be 0.745 mg guanidinoacetic acid/g kidney/7 hours, whereas the sample average activities for the protein-depleted rats (series 3, group 1 and group 2 in table 1) was 0.362 mg guanidinoacetic acid/g kidney/7 hours. Thus the carcasses of rats fed the protein-free diet had, in vitro, 48.7% (that is, 0.362/0.745) of the transamidinase activity of carcasses from rats fed the complete diet.

Because of the closeness of these relative rates, it was of interest to determine whether there was a substantial probability that the difference between the in vivo and in vitro rates, 43.5% and 48.7%, was due to chance. As with the previous analysis, the probability was obtained by referring to the normal curve of error the sample difference relative to its standard error. However, the calculation of the standard error in this situation was not routine. Specific notation is helpful. Let $\bar{y}$ represent the average creatine determination for a sample group of n rats. Similarly, denote by $\bar{x}$ the average transamidinase activity for such a sample. The 4 samples in this experiment will be labeled in the sequence: 1) protein-depleted diet, arginine-glycine injection; 2) protein-depleted diet, control glucose injection; 3) normal diet, arginine-glycine injection; and 4) normal diet, control glucose injection. For the in vivo analysis, the means are then $\bar{y}_1 = 244$, $\bar{y}_2 = 234$, $y_3 = 203$, $y_4 = 180$, and the rate of creatine synthesis may be denoted by

$$\frac{\bar{y}_1 - \bar{y}_2}{\bar{y}_3 - \bar{y}_4} \quad \text{(i.e., } \frac{10}{23} \text{ in the present case)}$$

The in vitro relative rate for the transamidinase activity is

$$\frac{(\bar{x}_1 + \bar{x}_2)/2}{(\bar{x}_3 + \bar{x}_4)/2}$$

wherein $\bar{x}_1 = 0.386$, $\bar{x}_2 = 0.339$, $\bar{x}_3 = 0.758$ and $\bar{x}_4 = 0.731$ (i.e., 0.362/0.745). Hence, the contrast between the in vitro and in vivo rates is

$$\frac{\bar{y}_1 - \bar{y}_2}{\bar{y}_3 - \bar{y}_4} - \frac{\bar{x}_1 + \bar{x}_2}{\bar{x}_3 + \bar{x}_4}$$

and it is the precision of this contrast, as measured by the standard error, that is required.

Taking in account the correlation between x and y in the data, the standard error formula for the contrast, as derived by propagation of error theory (20) for non-independent precision indices (21), is as shown in figure 3, wherein $r_{x_1y_1}$ designates the correlation coefficient (22) between the x_1 and y_1 values and similarly for the remaining correlations.

For the present data, calculation yields $SE_{contrast} = 0.17$. Hence relative to this standard error, the difference between the in vitro and in vivo rates is 0.29. The probability of a normal deviate of this absolute magnitude or greater arising by chance is 77%. That is, the rates do not differ significantly. Thus, it is substantiated statistically that the closeness of the rates 48.7% and 43.5% represents a good correlation between carcass transamidinase activities, in vitro, and creatine biosynthesis, in vivo. A more detailed treatment of the statistical analysis of these data appears elsewhere.[3]

In the third experiment, 24 male Holtzman rats weighing approximately 100 g were fed a complete diet for 10 days. Eleven of the rats were continued with the complete diet and 13 were fed a protein-free diet. Seven days later rats from each group were given intraperitoneal injections of 0.03 ml/g body weight of the solution of arginine plus glycine. The control rats were injected with the glucose solution. Each rat was given one injection per day for a period of 4 days. Urine was collected over the 4-day period in which the injections were given. The results are shown in table 1, series 4. The amount of creatine synthesized from the exogenous glycine plus arginine in the rats fed the complete diet was estimated to be 259 − 219 or 40 mg creatinine/100 g carcass. The rats fed the protein-free diet synthesized 266 − 250 or 16 mg creatinine/100 g carcass. The transamidinase activities, in vitro, of the carcasses from the rats fed the complete diet, averaged over both injections, were estimated at 0.49, whereas the sample average activities for the protein-depleted rats were 0.23. Again there was a good correlation between carcass transamidinase activities and biosynthesis of creatine, the relative rates being 46% and 40%, respectively.

[3] McHugh, R. B., J. F. Van Pilsum and F. W. Briese Validation of an in vitro enzyme assay indirectly measuring an in vivo biosynthesis. (Submitted for publication).

$$SE^2_{contrast} = \left(\frac{\bar{y}_1 - \bar{y}_2}{\bar{y}_3 - \bar{y}_4}\right)^2 \left[\frac{SE^2(\bar{y}_1) + SE^2(\bar{y}_2)}{(\bar{y}_1 - \bar{y}_2)^2} + \frac{SE^2(\bar{y}_3) + SE^2(\bar{y}_4)}{(\bar{y}_3 - \bar{y}_4)^2}\right] + \left(\frac{\bar{x}_1 + \bar{x}_2}{\bar{x}_3 + \bar{x}_4}\right)^2 \left[\frac{SE^2(\bar{x}_1) + SE^2(\bar{x}_2)}{(\bar{x}_1 + \bar{x}_2)^2} + \frac{SE^2(\bar{x}_3) + SE^2(\bar{x}_4)}{(\bar{x}_3 + \bar{x}_4)^2}\right]$$

$$- 2\left(\frac{\bar{y}_1 - \bar{y}_2}{\bar{y}_3 - \bar{y}_4}\right)\left(\frac{\bar{x}_1 + \bar{x}_2}{\bar{x}_3 + \bar{x}_4}\right)\left[\frac{r_{x_1 y_1}\, SE(\bar{x}_1)SE(\bar{y}_1) - r_{x_2 y_2}\, SE(\bar{x}_2)SE(\bar{y}_2)}{(\bar{x}_1 + \bar{x}_2)(\bar{y}_1 - \bar{y}_2)} + \frac{r_{x_3 y_3}\, SE(\bar{x}_3)SE(\bar{y}_3) - r_{x_4 y_4}\, SE(\bar{x}_4)SE(\bar{y}_4)}{(\bar{x}_3 + \bar{x}_4)(\bar{y}_3 - \bar{y}_4)}\right]$$

Fig. 3 Variance of the contrast between the carcass transamidinase activities, in vitro, and rates of creatine synthesis, in vivo.

Rats fed the complete diet and given injections of arginine plus glycine synthesized daily an amount of creatine which was 7% (series 2) and 5% (series 4) of the total carcass creatine. Rats fed the protein-free diets synthesized about one-half these amounts (series 3 and 4). It is not known whether the amount of creatine synthesized would have been greater if the dose of arginine plus glycine had been larger than was used in these 2 experiments. Also, is is not known whether the incorporation of a methyl donor, such as methionine, into the solutions of arginine-glycine would have increased the amount of creatine that was synthesized. However, it appears that the amount of creatine synthesized per day after the injections of arginine plus glycine was greater than the amount which equals 2% of the total carcass creatine. Bloch et al. (23) have calculated that a normal adult rat synthesized daily an amount of creatine equal to 2% of the total carcass creatine.

It is believed that the rats synthesized amounts of creatine greater than an amount equivalent to 2% of the total carcass creatine because they were given large doses of arginine and glycine. Bloch et al. (23) did not use large doses of arginine plus glycine in their investigations. Also, possibly the rates of creatine synthesis may be greater in young than in adult animals which were used by Bloch et al. (23). It is also possible that the reason for the relatively large rates of creatine synthesis after the administration of arginine and glycine was that the transamidinase was more saturated with its substrates than it was without the injections. It is not known whether the administration of arginine plus glycine resulted in a transitory increase in the transamidinase activities. If the injection of arginine plus glycine did alter the transamidinase activities, it could only have been transitory, since the carcass activities of the rats that received the injections were similar to the activities of the carcasses from the rats that were given injections of glucose (table 1).

Of the 9 tissues present in the carcass homogenate that have transamidinase activity, only pancreas and kidney have been

shown to be low in protein depletion (6). Kidney and pancreas from rats fed a protein-free diet for 5 days had 32% and 55%, respectively, of the activities found in rats fed a complete diet (6). Longer periods of protein depletion (7–9 days) produce still further reduction (15–20% of normal) in kidney activities. There is no doubt that the low kidney and pancreas activities were a factor in the low carcass activities of the rats fed the protein-free diet. In this study, when rats were given large doses of arginine plus glycine, there was an excellent correlation between carcass transamidinase activities and rates of creatine synthesis, in vivo. This was certainly a better correlation than would have been found between kidney transamidinase activities and rates of creatine biosynthesis. One possible reason for this good correlation may have been that in both determinations (the rate of creatine biosynthesis and the carcass transamidinase activities) the transamidinase was saturated with its substrates.

It has not been possible to determine which tissue or tissues are a major factor(s) in the synthesis of guanidinoacetic acid, in vivo. Therefore alterations in any one tissue transamidinase activity cannot be identified as alterations in rates of creatine biosynthesis, as has been done by others (9). The fact that we found the good correlation between the carcass transamidinase activities and creatine biosynthesis is considered as evidence that guanidinoacetic acid synthesis, in vivo, is not restricted to kidney.

The problem should be investigated further by determining rates of creatine synthesis, in vivo, with the use of the trace amounts of isotope labeled creatine precursors. The results of the present work and of the isotope incorporation studies should be helpful in interpreting the meaning of transamidinase activities, in vitro, as far as their effects on the biosynthesis of creatine are concerned. At any rate, at present, there is no justification for identifying alterations in kidney transamidinase activities, in vitro, as the "metabolic control of creatine biosynthesis" (9).

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Lack of Effect of Thiamine Deficiency on Oxidation of Methylglyoxal-^{14}C in the Rat

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ABSTRACT The effect of thiamine deficiency on the in vivo oxidation of methylglyoxal-1,3-^{14}C and methylglyoxal-2-^{14}C to $^{14}CO_2$ was studied in the rat. The rapidity with which the ^{14}C label appeared in the expired CO_2 favors the interpretation that methylglyoxal metabolism is not impaired in the thiamine-deficient rat. The appearance of ^{14}C label in liver glycogen and urine keto acids also indicated that it rapidly entered the classical metabolic pathways. The data suggest a rapid transformation of methylglyoxal to pyruvate and entrance into the Krebs cycle.

The reported occurrence of methylglyoxal as an aberrant metabolite in vitamin B_1 deficiency has held the interest of numerous investigators in the field of carbohydrate metabolism.

This compound was first detected in the body fluids of patients with beriberi and the observation was made that there was a more marked toxemia in the presence of appreciable quantities of methylglyoxal (1). It has been reported frequently that an accumulation of methylglyoxal exists in the thiamine-deficient animal (2, 3). Information on the metabolic origin of methylglyoxal is lacking and what little exists is controversial. Early investigators (4, 5) believed its appearance and existence to be a non-enzymatic artifact of muscle metabolism. Others (1, 6) believed it to be real and to be a toxic product derived from carbohydrate metabolism in the absence of thiamine pyrophosphate. Two independent studies are not in accordance with these results and have failed to confirm its suggested role in carbohydrate metabolism (7, 8).

Because of the widespread distribution of the enzyme glyoxalase in the cells of both vertebrates and invertebrates it appears that the natural substrate for glyoxalase is methylglyoxal and a possible role in the carbohydrate scheme exists for this compound.

Although the metabolic pathways of methylglyoxal have not been defined clearly, it has been established that methylglyoxal is metabolized rapidly in the presence of glutathione by the enzyme glyoxalase which consists of 2 components. The first component acts with glutathione to form a condensation product. This condensation product is then catalyzed by the second glyoxalase component to reduced glutathione and D-lactate (9). Recent investigations show that methylglyoxal may play a significant role in cellular metabolism as well as a prominent role as an intermediate in the aminoacetone cycle (10). At present this is the only known enzymatic synthesis of methylglyoxal in the animal organism and results through degradation of threonine through aminoacetone to methylglyoxal. To clarify the fate of methylglyoxal in the thiamine-deficient rat, both methylglyoxal-1, 3-^{14}C and methylglyoxal-2-^{14}C were prepared and studied to determine the rate and mechanism of oxidation. The following study provides experimental data on the ability of thiamine-deficient animals to metabolize methylglyoxal and on the pathways involved compared with a control group.

EXPERIMENTAL PROCEDURES

Animals. Rats of both sexes (siblings of a Sprague-Dawley-descended strain) that have been propagated in our laboratory in a random-bred closed colony program for 4 years were used as experimental animals.

Animals whose initial weights ranged from 81 to 121 g were divided into pairs according to sex and weight. The animals

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were maintained in individual metabolic cages.[1] A pair-feeding regimen was used during the entire study.

Thiamine deficiency was induced in the animals by feeding a commercial thiamine-deficient diet.[1] The control animals were fed a similar diet to which thiamine·HCl was added in the quantity of 1 μg/g of diet. Thiamine-deficient animals and their paired controls were maintained with the diets for a period of 5 weeks. On the completion of this period, all animals fed the thiamine-deficient diet showed an advanced "thiamine deficiency syndrome" characterized by anorexia, inanition (a 20–30% loss of body weight from peak weight), loss of muscular coordination, and general debility.

MATERIALS AND METHODS

Methylglyoxal-1, 3-^{14}C ($^{14}CH_3CO^{14}CHO$) and methylglyoxal-2-^{14}C ($CH_3{}^{14}COCHO$) were prepared by the oxidation of ^{14}C-labeled acetone (obtained from Nuclear-Chicago Corporation) with selenium dioxide. The crude methylglyoxal was purified by fractional distillation under a nitrogen atmosphere and concentrated under reduced pressure by the method of Brum (11) and Winteringham.[2]

The distribution of the radioactivity in the expired CO_2, liver glycogen and in urine metabolites in the rat was measured after injection with methylglyoxal-1, 3-^{14}C and methylglyoxal-2-^{14}C. All animals prior to being given ^{14}C-labeled methylglyoxal were fed 5 ml of a 25% glucose priming solution by mouth to activate the Embden Meyerhof and Krebs cycles and to enhance glycogen formation in the liver. The animals were then injected intraperitoneally with 2.28 μCi (0.25 μmole) of methylglyoxal-1,3-^{14}C or 1.64 μCi (0.30 μmole) of methylglyoxal-2-^{14}C made up in 1 ml of Na_2HPO_4-KH_2PO_4 Sorenson buffer (pH 7.48)/100 g of body weight. The animals then were placed immediately in a glass metabolic chamber swept with a slow stream of air and they remained there for 6 hours. Twelve one-half-hour samples of expired CO_2 were collected in 100 ml of 1 N NAOH and the $^{14}CO_2$ activity was assayed by standard procedures (12). Urine collected during this period was saved for analysis by paper chromatography (13). On completion of the collection period the animals were anesthetized with 5 mg of pentobarbital sodium, the liver was rapidly extirpated, glycogen extracted, and the radioactivity determined (14).

Samples of barium carbonate, glycogen, and urine metabolite derivatives were all plated to infinite thinness on preweighed stainless steel planchets and the radioactivity was determined by a Picker proporional flow counter used with a six direct reading plug-in decade clinical scaler.

RESULTS AND DISCUSSION

The results of the oxidation of methylglyoxal-1,3-^{14}C and of methylglyoxal-2-^{14}C by both thiamine-deficient and control rats are graphically represented and interpreted in figures 1 and 2. Figure 1 shows that the thiamine-deficient rats metabolized methylglyoxal-1, 3-^{14}C more rapidly and to a greater extent than the control rats after the first 1.5 hours. In the thiamine-deficient animals, 71.5% of the administered dose was utilized and appeared as ^{14}C in CO_2 at the end of a 6-hour period. In the controls, 52.7% of the dose was utilized and appeared as ^{14}C in the expired CO_2 during the same time period. The difference was significant at $P < 0.05$. Figure 2 shows that the controls metabolized more of the methylglyoxal-2-^{14}C than the thiamine-deficient animals. The controls in this study utilized 87.2% of the total dose administered and the thiamine-deficient rats, 75.3%. However, the difference was not significant.

The total counts per minute per milligram of carbon expired as CO_2 by the thiamine-deficient rats shown in figure 3 was greater for all periods following the first hour than by the control group. However, the total metabolism of carbon com-

[1] Percentage composition of thiamine-deficient diet; vitamin test casein, 18; sucrose, 68; vegetable oil, 10; salt mixture no. 2, USP XIII, 4; and vitamin diet-fortification mixture which contained per 45.5 kg of diet: (in grams) vitamin A conc (200,000units/g), 4.5; vitamin D conc (400,000 units/g), 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; Ca pantothenate, 3.0; and (in milligrams) biotin, 20.0; folic acid, 90.0; vitamin B_{12}, 1.35. Test diet was obtained from General Biochemicals, Chagrin Falls, Ohio.

[2] Personal communication from F. P. Winteringham, Head, Biochemistry Department, Agriculture Research Council, England, 1963.

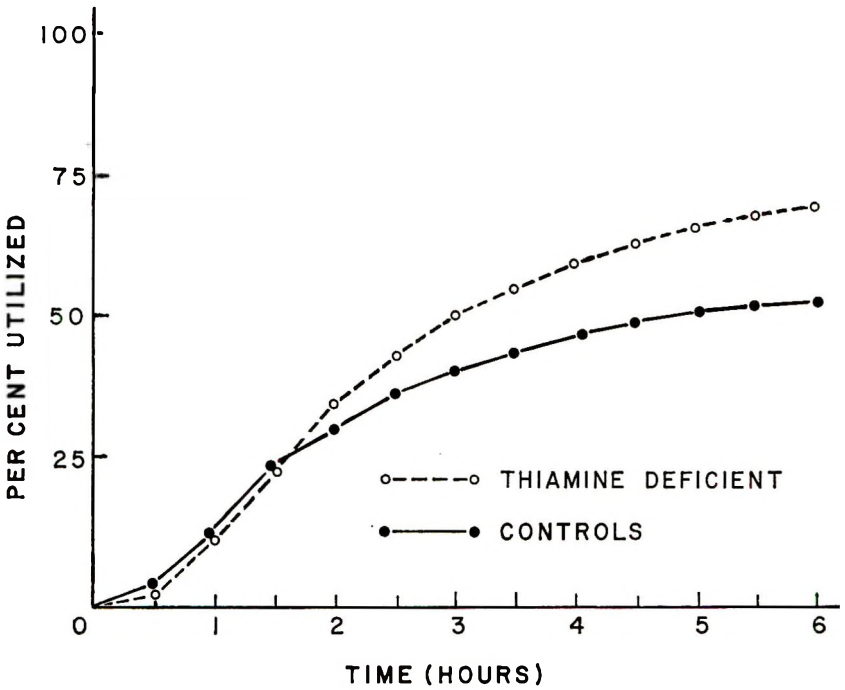

Fig. 1 Cumulative percentage of total methylglyoxal-1,3-^{14}C expired as $^{14}CO_2$. Ten animals were started in each group. One deficient animal died during the sixth period. Animals were injected intraperitoneally with 2.28 μCi (0.25 μmoles)/100 g body weight.

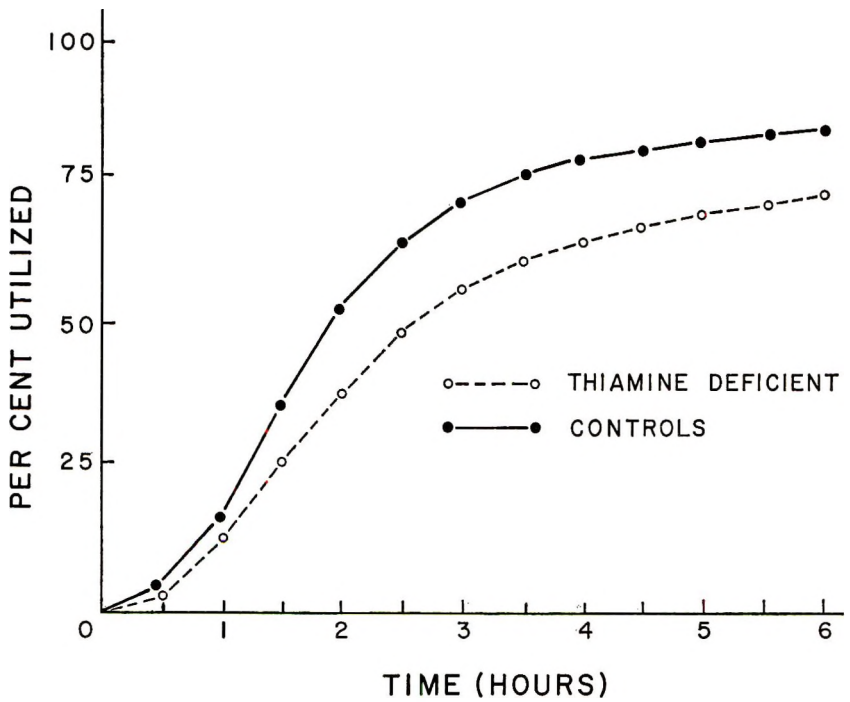

Fig. 2 Cumulative percentage of total methylglyoxal-2-^{14}C expired as $^{14}CO_2$. Eleven animals were started in each group. One deficient animal died during the eighth period and one control animal died immediately after receiving injection of methylglyoxal-2-^{14}C. Animals were injected intraperitoneally with 1.64 μCi (0.30 μmoles)/100 g body weight.

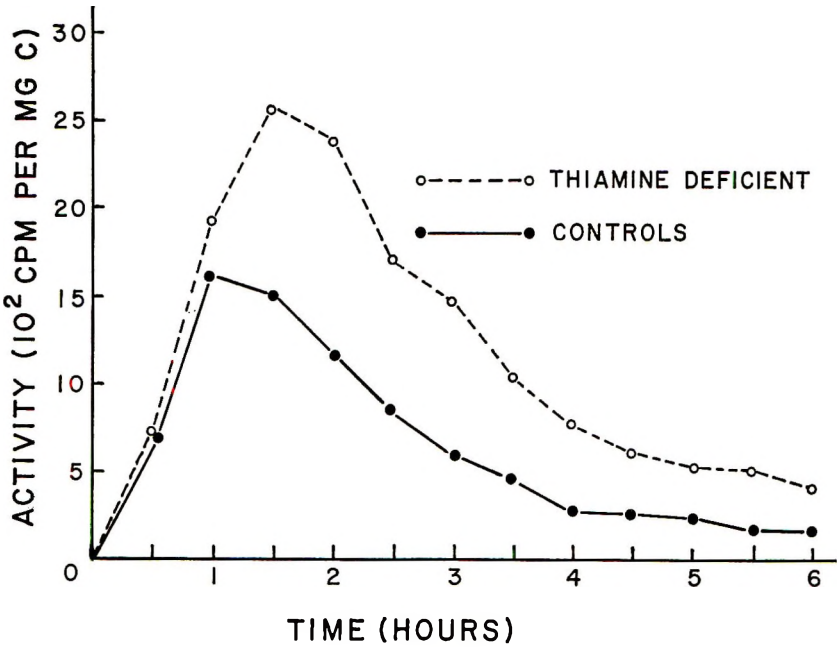

Fig. 3 Rate of excretion of methylglyoxal-1,3-^{14}C in expired CO_2, total counts/min/mg of carbon. Ten animals were started in each group. One deficient animal died during the sixth period. Animals were injected intraperitoneally with 2.28 μCi (0.25 μmoles)/100 g body weight.

pounds exhaled as CO_2 was greater in the controls than in the thiamine-deficient rats administered methylglyoxal-1,3-^{14}C. It appeared that the pair-fed controls utilized more of the glucose (fed to all rats injected with methylglyoxal-^{14}C) than did the thiamine-deficient rats. The fact that thiamine pyrophosphate (TPP) was still available in the pair-fed rats and was not as plentiful in the thiamine-deficient rats would account for the greater CO_2 production from glucose via the Krebs cycle. The conversion of methylglyoxal in the thiamine-deficient rat to a dicarboxylic acid intermediate is offered as a possible alternate pathway to the Krebs cycle. Following the conversion of methylglyoxal to pyruvate the formation of a 4-carbon dicarboxylic acid, malate or oxalacetate takes place in the absence of thiamine pyrophosphate by the condensation of pyruvate with CO_2 (15).

Pyruvate oxidation studies using ^{14}C-labeled pyruvate in both thiamine-deficient and control rats under similar experimental conditions have been reported (16) and favor a dicarboxylic acid intermediate prior to admission into the Krebs cycle. However, Jones and de Angeli (17) in their studies on the oxidation of ^{14}C-labeled lactate and pyruvate in the thiamine-deficient animal noted that in respect to production of ^{14}C-labeled $^{14}CO_2$ there was no difference between deficient and control animals and that clinical manifestations resulting from thiamine-deficiency were not the result of a specific failure of the animal to decarboxylate pyruvate.

The counts per minute per milligram of carbon for the 2 groups receiving methylglyoxal-2^{14}C coincided. Production of CO_2 (from all available carbon sources including the methylglyoxal-2-^{14}C) was diminished in the thiamine-deficient rats. This is shown in figure 4.

The appearance of ^{14}C label in liver glycogen and urine keto acids indicated that methylglyoxal rapidly enters the classic metabolic pathways. Paper chromatograms of the dinitrophenylhydrazine derivatives of neutral carbonyl compounds

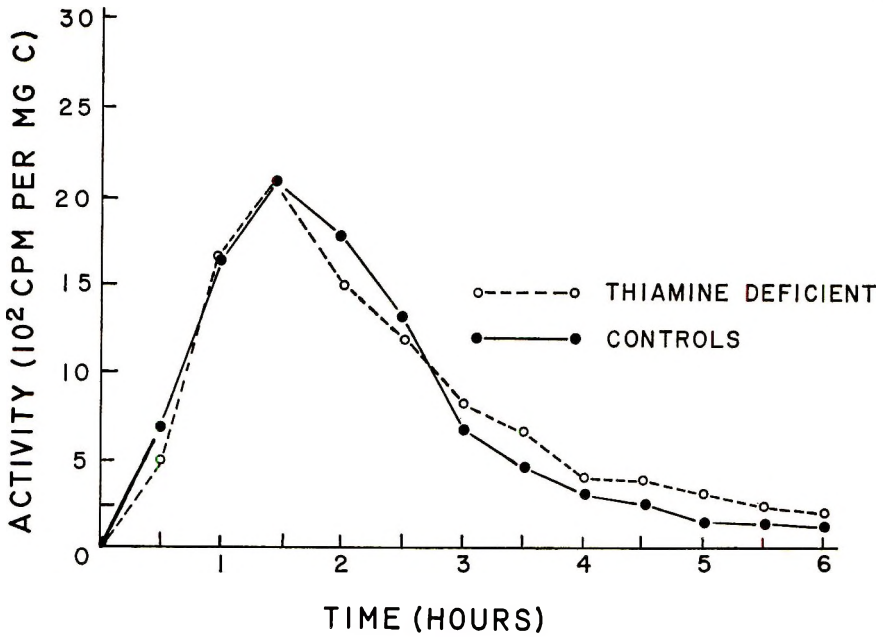

Fig. 4 Rate of excretion of methylglyoxal-2-^{14}C in expired CO_2, total counts/min/mg of carbon. Eleven animals were started in each group. One deficient animal died during the eighth period and one control animal expired immediately after receiving injection of methylglyoxal-2-^{14}C. Animals were injected intraperitoneally with 1.64 μCi (0.30 μmoles) /100 g body weight.

and acidic keto acids present in rat urine collected for 6 hours after both methylglyoxal-1,3-^{14}C and methylglyoxal-2-^{14}C administration showed ^{14}C activity in both ketoglutarate and pyruvate as well as in an unidentified fraction having an R_F value of 0.49. This is represented in figures 5 and 6. The dinitrophenylhydrazone of pyruvic acid gives 3 spots. These are three isomeric forms of the hydrazones of this compound and have been observed previously and reported (18). The higher levels of α-ketoglutarate observed in the urine of thiamine-deficient rats compared with controls are in agreement with elevated levels reported by Wright and Scott (19). They also reported that in the thiamine-deficient rat pyruvate is the predominant keto acid in blood, whereas ketoglutarate predominates in the urine.

In advanced thiamine deficiency as well as other vitamin deficiency states transaminase reactions are depressed

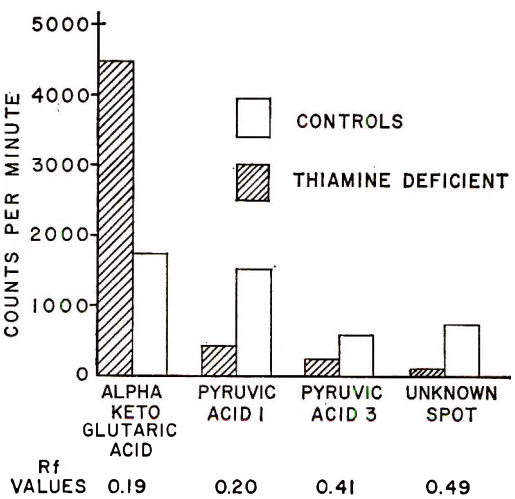

Fig. 5 Activity of rat urine keto acid dinitrophenylhydrazine derivatives after the administration of methylglyoxal-1,3-^{14}C. Urine was collected for 6 hours following intraperitoneal injection of 2.28 μCi (0.25 μmoles)/100 g body weight.

(20). The rate of amination of α-ketoglutarate from an amino donor to form glutamate is decreased. The endogenous α-ketoglutarate pool is extended and the elevated α-ketoglutarate levels are reflected in the urine. In the pair-fed controls, this is also observed but to a much lesser degree.

The presence of glyoxylate in the urine of both controls and thiamine-deficient rats has been reported by Liang (21). He concluded that the formation of glyoxylate was chiefly the result of deamination of glycine but could also arise from oxidation and demethylation of methylglyoxal. Table 1 gives the results of the incorporation of methylglyoxal-^{14}C into liver glycogen. The deficient animals incorporated less of the ^{14}C label than their controls and both the liver size and weight and glycogen content were markedly reduced.

The present study showed that both the thiamine-deficient and control animals readily metabolized ^{14}C-labeled methylglyoxal and the rapidity with which the ^{14}C label appeared in the expired CO_2 favored the interpretation that methylglyoxal metabolism is not impaired in the thiamine-deficient rat. An alternate pathway for the oxidation of methylglyoxal in the thiamine-deficient rat is suggested

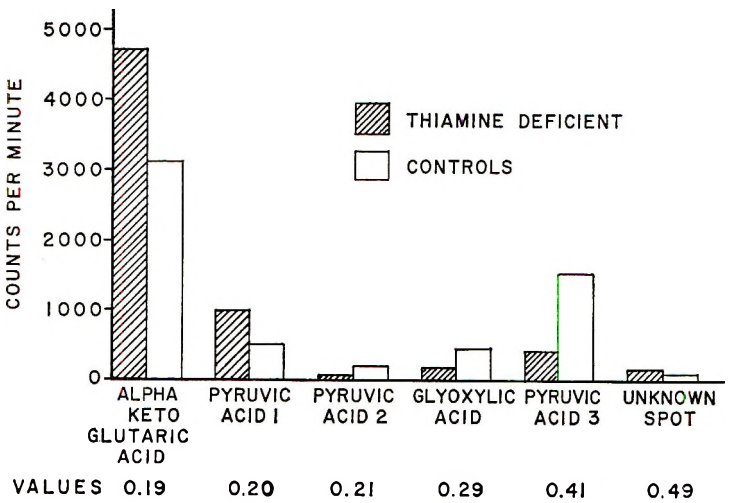

Fig. 6 Activity of rat urine keto acid dinitrophenylhydrazine derivatives after the administration of methylglyoxal-2-^{14}C. Urine was collected for 6 hours following intraperitoneal injection of 1.64 μCi (0.30 μmoles)/100 g body weight.

TABLE 1

In vivo incorporation of ^{14}C-methylglyoxal [1] into liver glycogen of thiamine-deficient and control animals

Status of animal	Liver wt [2]	Glycogen wt	Specific activity of liver glycogen
	g	*mg*	*count/min/mg*
	Methylglyoxal-1,3-^{14}C		
Thiamine-deficient	4.8	144	53
Control	7.5	300	126
	Methylglyoxal-2-^{14}C		
Thiamine-deficient	5.2	140	100
Control	6.0	225	188

[1] Dose injected: 2.28 μCi (0.25 μmole) methylglyoxal-1,3-^{14}C/100 g body weight; 1.64 μCi (0.30 μmole) methylglyoxal-2-^{14}C/100 g body weight.

[2] Livers extirpated from both groups of animals 6 hours after receiving ^{14}C-labeled methylglyoxal.

since rats in a state of thiamine deficiency oxidized more of the labeled methylglyoxal-1, 3-^{14}C than their controls. Oxidation of methylglyoxal normally occurring via the Krebs cycle is evidently impaired in the thiamine-depleted rat, resulting in a greater pool to be oxidized by an alternate route. In general where a significant difference existed in the rate of methylglyoxal oxidation between the thiamine-deficient and control rat it was in favor of a greater rate of oxidation or utilization in the deficient rat. The data further suggest that thiamine was not essential for the metabolic pathway studied and in the deficient animal lack of thiamine resulted in a larger labeled pool available for oxidation than existed in the control. It is possible that the evaluation of the radioactivity in the expired CO_2 was not a measure of the oxidation of the radioactive carbon-1 of methylglyoxal-1, 3-^{14}C but only of carbon-3. Since the integrity of the tricarboxylic acid cycle is necessary for oxidation of both carbon-2 and carbon-3 of pyruvate, it might be assumed that the metabolic fate of carbon-2 and carbon-3 of methylglyoxal also enter an identical pathway. This could occur following conversion to pyruvate and admission into the tricarboxylic acid cycle via oxidative decarboxylation to acetyl-CoA for by condensation with CO_2 to form a dicarboxylic acid intermediate prior to further cycling. It is known that the CO_2 of the carboxyl group of lactic acid can be replaced by the CO_2 of tissue cells by the carboxylation of pyruvate forming malate and oxalacetate. A comparable exchange involving the labeled aldehyde carbon of methylglyoxal-1, 3-^{14}C might also occur. The data presented, however, favor an interpretation of a minimum of randomization and exchange taking place. Since 71.5% of the labeled carbon appeared in the respiratory CO_2 of the thiamine-deficient rats and 52.7% in the controls, the integrity of the Krebs cycle is necessary for the oxidation of carbon-3. Additional confirmation was obtained by preparing methylglyoxal-2-^{14}C and studying its metabolism in thiamine-deficient and control rats. Presumably the proper function of the Krebs cycle is necessary for the biological oxidation of the radioactive carbon of this compound. The activity of both pyruvic acid α-ketoglutaric acid decarboxylase is dependent on thiamine pyrophosphate (TPP) as a coenzyme. A difference in total counts per minute or more specifically in counts per milligram of carbon might therefore be anticipated between the thiamine-deficient and control animals. The data show that the control rats metabolized a greater amount of methylglyoxal-2-^{14}C than the thiamine-deficient rats and are presented in figure 2. The differences, however, were not significant.

The data obtained from these experiments favor the conclusion that following a rapid conversion to pyruvate the major pathway for methylglyoxal metabolism is via the Krebs cycle, and provides conclusive evidence that methylglyoxal does not accumulate in the thiamine-deficient rat.

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Initial Effects of Amino Acid Imbalance in the Rat [1]

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ABSTRACT In an attempt to distinguish the initial effects of an amino acid imbalance from the indirect effects of the depressed food intake, several biochemical changes were determined in protein-depleted rats in short-term experiments. The rats were fed for 24 hours an imbalanced diet, made by adding 3.1% of an amino acid mixture lacking threonine to a basal diet containing 10% wheat gluten supplemented with 0.8% of lysine and 0.1% of DL-threonine. In pair-feeding experiments it was observed that the protein, amino acid N and threonine content in liver of rats fed the imbalanced diet was higher than in those fed the basal diet. Relative high levels of liver protein concentration in protein-depleted rats after feeding the imbalanced diet were also observed in ad libitum experiments. These observations support the assumption that the imbalance could act initially, stimulating the liver protein synthesis. An increase in liver weight correlated with a high glycogen content was also observed in protein-depleted rats fed the imbalanced and the corrected diet (imbalanced plus 0.1% DL-threonine) both in ad libitum or in the pair-feeding experiments. These observations indicate the utilization of the excess of amino acids provided by the amino acid mixture for glyconeogenesis. As a consequence of the changes in liver and plasma concentration of threonine and amino acid N, some of the ratios that reflect their relative proportions were markedly altered. The possibility that the depression in food intake characteristic of the imbalance could be related to those changes is discussed.

Depression in food intake, retarded growth and changes in food preference were the first effects reported to appear in rats fed diets in which an imbalance of amino acids was created (1, 2). Later, it was likewise observed that the plasma amino acid pattern was markedly altered within a short time after the ingestion of an imbalanced diet (3, 4). As these changes began at the time when the decrease in food intake was first detected, it was assumed that some relationship might exist between the altered blood amino acid pattern and the decrease in food intake (4).

Further studies demonstrated that concurrently with those effects some changes occurred in the liver of depleted and non-depleted rats after feeding for 2 weeks an imbalanced diet containing 10% of wheat gluten (5). These observations raised the question of whether these changes were direct effects of the imbalance or were only the consequence of a depressed food intake.

In the present paper we describe some biochemical changes observed in tissues of protein-depleted rats after feeding an imbalanced diet for 24 hours. As suggested by Harper et al. (6) these changes that occur in a short period of time can be considered as initial and direct effects of the imbalance, effects that are different from those observed after long-term experiments that could be the indirect effects of an adaptation of the organism to adverse conditions. The relationship that may exist between some of these changes and the depression in food intake is also discussed here.

EXPERIMENTAL

Male rats of the Wistar strain, having an average weight of 80 to 85 g, were used in these experiments. All rats were depleted by feeding them a protein-free diet for 7 days. Animals that lost approximately 15 to 17 g were selected and separated into groups of 6 rats each.

The rats were housed in individual suspended cages with screen bottoms and water was offered ad libitum. The diets

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TABLE 1

Composition of diets

	Basal (A)	Imbalanced (B)	Corrected (C)	Corrected (D)	Basal (E)
	%	%	%	%	%
Wheat gluten [1]	10	10	10	10	10
L-Lysine [2]	0.80	0.80	0.80	0.80	0.80
DL-Threonine	0.10	0.10	0.20	0.60	0.60
Amino acid mixture [3]	—	3.10	3.10	3.10	—
Minerals [4]	5	5	5	5	5
Vitamin mixture [4]	0.25	0.25	0.25	0.25	0.25
Choline chloride	0.15	0.15	0.15	0.15	0.15
Corn oil [5]	5	5	5	5	5
Dextrin [6]	78.70	75.60	75.50	75.10	78.20
Total protein content [7]	9.28	12.38	12.48	12.88	9.78
Total L-threonine content	0.25	0.25	0.30	0.50	0.50

[1] Containing 13.4% N (83.8% of protein), 2% of threonine and 1% of lysine.
[2] As L-lysine·HCl.
[3] The amino acid mixture provided in % in the diet: L-arginine·HCl, 0.2; L-lysine·HCl, 0.4; DL-isoleucine, 0.4; L-leucine, 0.3; DL-methionine, 0.4; DL-phenylalanine, 0.2; L-histidine·HCl, 0.4; DL-tryptophan, 0.4; and DL-valine, 0.4.
[4] Harper, A. E. (7).
[5] Fat-soluble vitamins were included in the corn oil (7).
[6] Moist cornstarch heated at 121° in an autoclave for 3 hours.
[7] Including added amino acids.

were similar to those described in a previous paper (5) and are outlined in table 1.

In all experiments, the animals were fed the experimental diets for 24 hours. A control group continued the consumption of the protein-free diet for another 24 hours after the depletion period. In the ad libitum experiments food intake was measured. In the pair-feeding experiments the 2 groups of animals receiving the basal and corrected diets were pair-fed with those receiving the imbalanced diet which was fed ad libitum. Therefore, in the pair-feeding experiments the basal and the imbalanced groups had identical intakes of threonine, the limiting amino acid for growth.

In all experiments rats were weighed 4 hours after the feeding period; they were then killed and plasma samples and organs were taken for analysis.

Collection of material. The rats were anesthesized with ether, and blood was withdrawn by heart puncture. The blood of each group of 6 rats was pooled and the plasma was separated from the heparinized blood by centrifugation. Aliquots of plasma samples were deproteinized according to the method described below for the subsequent determination of urea, and amino acid N. Whole plasma was used for threonine determination.

After weighing the whole liver of exsanguinated rats, a sample was placed in 10% perchloric acid and blended in a Virtis homogenizer; suitable aliquots of this suspension were used for protein determination. A large sample was frozen for subsequent lipid determination. The remaining liver was divided into 2 pieces; one was homogenized in 30% KOH for glycogen determination and the other was homogenized in distilled water for threonine and amino acid N evaluation.

The right gastrocnemius muscle was weighed, placed in 10% perchloric acid and blended in a Virtis homogenizer. Aliquots of this suspension were used for protein determination.

Chemical analysis. Tissue protein was determined by the method of Marenzi et al. (8), liver lipid content by the procedure of Sidransky and Baba (9), liver glycogen according to Krisman (10) and threonine by the method of Neidig and Hess (11) after the microdiffusion technique of Winnick (12). Urea was determined by the method of Archibald as modified by Ratner (13) and the determination of amino acid N was performed by the method of Danielson as described by Hawk et al. (14).

For the determination of urea and amino acid N, the samples of plasma and liver homogenates were previously depro-

teinized with zinc sulfate-sodium hydroxide.

RESULTS

Ad libitum experiments

Body and organ weights and food intake. Table 2 shows the food intake and changes in body and organ weights (liver and gastrocnemius muscle) of protein-depleted rats fed the experimental diets ad libitum for 24 hours. As would be expected, the rats fed imbalanced diet B had a lower consumption than those receiving the basal diet. The average weight of the imbalanced rats was slightly reduced after the experimental period; in contrast, rats fed the basal diet regained approximately half of the weight they had lost during the depletion period.

The group fed corrected diet C gained more weight than the basal diet group, indicating that the imbalance was wholly overcome by the addition of 0.1% of DL-threonine. Protein-depleted rats fed corrected diet D, containing a high concentration of DL-threonine, showed a gain in weight and a food intake somewhat greater than those fed basal diet E. Hence, this result confirms that no effects of the imbalance occur when the basal diet contains a large amount of the limiting amino acid.

The gain in liver weight of rats fed balanced diets over that of animals fed the protein-free diet appears to be related to the quantity of protein consumed. The highest liver weights were observed in groups fed corrected diets D and C, that had protein intakes of 1.47 and 1.37 g, respectively. The increase in weight was less for groups fed basal diets A and E, with protein intakes of 1.18 and 1.05 g, respectively. On the contrary, this relationship was not observed in rats fed imbalanced diet B, that had a liver weight not significantly different from that of animals receiving the basal diet A, even though their total protein intake was only of 0.80 g.

Biochemical changes. A summary of the liver protein, glycogen and lipid content of the animals fed the experimental diets ad libitum is shown in table 2.

The increase in liver protein content in the group fed imbalanced diet B was similar to that for the groups receiving more balanced diets, even though the protein intake of the imbalanced group was greatly reduced in comparison with that of the others.

The liver lipid content was essentially the same in all the groups fed the experimental diets and similar to that of the protein-free group.

The gastrocnemius muscle protein content in rats fed imbalance diet D was similar to that of the protein-free diet and significantly different from the value for the basal group. No significant differences in that component were observed between the basal group, and those fed diets C, D and E.

Pair-feeding experiments

Body and organ weights. The results obtained in these experiments are shown in table 2. Protein-depleted rats fed equal amounts of the basal or the imbalanced diet (6.5 g) showed a slight decrease in weight after the experiment but those pair-fed the corrected diet regained 2.3 g of the weight lost during the depletion period.

The mean wet-weight of the liver was significantly increased in the rats fed the imbalanced and the corrected diets as compared with those fed the basal diet. Here again, as observed in the ad libitum experiments, the gain in liver weight appears to be related to the amount of protein ingested. The gastrocnemius muscle weight was essentially the same in the 3 groups and similar to that of the protein-free group.

Biochemical changes. In protein-depleted rats killed 4 hours after the termination of the 24-hour pair-feeding experiments, the total amount of liver protein was significantly higher in rats receiving the imbalanced diet than in those pair-fed the basal diet, despite the intake of threonine, the first limiting amino acid of the diet, being the same for both groups. Moreover, the protein content in the liver of rats fed the imbalanced diet was similar to that of the rats pair-fed the corrected diet, despite their lower intake of threonine.

The liver glycogen content was similar in groups fed the imbalanced and the

TABLE 2

Weight and composition of liver and gastrocnemius muscle of depleted rats fed the experimental diets for 24 hours [1]

Diet	Food intake	Change in body wt	Liver				Gastrocnemius muscle	
			Wet wt	Protein	Lipid	Glycogen	Wet wt	Protein
	g/rat/24 hr	*g*	*g*	*mg/liver*	*mg/liver*	*mg/liver*	*g*	*mg/muscle*
Ad libitum experiments								
Basal A (10% wheat gluten) [2]	12.8	+6.3 ± 0.2 [3]	4.000 ± 0.090	570 ± 28	220 ± 18	290 ± 23	334 ± 21	64.0 ± 2.5
Imbalanced B (A + 3.1% AA mix) [4]	6.5	−0.5 ± 0.1 [5]	3.630 ± 0.100	551 ± 10	240 ± 18	235 ± 32	325 ± 9	52.0 ± 1.0 [5]
Corrected C (B + 0.1% DL-threonine)	11.0	+7.8 ± 0.2 [5]	4.463 ± 0.110 [5]	545 ± 10	230 ± 15	406 ± 20 [5]	300 ± 5	58.0 ± 1.5
Corrected D (B + 0.5% DL-threonine)	11.3	+8.6 ± 0.3 [5]	4.842 ± 0.135 [5]	596 ± 15	240 ± 25	464 ± 20 [5]	335 ± 12	63.5 ± 2.0
Basal E (A + 0.5% DL-threonine)	10.8	+8.0 ± 0.5 [5]	3.910 ± 0.125	532 ± 23	210 ± 10	305 ± 28	328 ± 10	62.5 ± 2.0
Protein-free	—	—	2.795 ± 0.095 [5]	402 ± 15 [5]	180 ± 20	190 ± 25 [5]	320 ± 7	52.0 ± 2.6 [5]
Pair-feeding experiments								
Basal A (10% wheat gluten) [2]	6.5 [6]	−0.2 ± 0.1	2.920 ± 0.120	490 ± 9	185 ± 15	125 ± 20	298 ± 15	50.5 ± 2.0
Imbalanced B (A + 3.1% AA mix) [4]	6.5	−0.5 ± 0.1	3.630 ± 0.100 [5]	551 ± 10 [5]	240 ± 18	235 ± 32 [5]	325 ± 9	52.0 ± 1.0
Corrected C (B + 0.1% DL-threonine)	6.5 [6]	+2.3 ± 0.1 [5]	3.850 ± 0.105 [5]	555 ± 17 [5]	220 ± 22	222 ± 30 [5]	300 ± 10	52.3 ± 2.0
Protein-free	—	—	2.795 ± 0.095	402 ± 15 [5]	180 ± 20	190 ± 25	298 ± 15	50.5 ± 2.0

[1] Rats were weighed and killed 4 hours after the experimental period.
[2] Supplemented with 0.8% of L-lysine as L-lysine·HCl, and 0.1% of DL-threonine.
[3] Mean value ± SE of mean.
[4] For composition, see footnote 3, table 1.
[5] Highly significantly ($P < 0.01$) different from basal group mean of each set of experiments.
[6] Pair-fed with the group fed the imbalanced diet B.

corrected diet and significantly higher than that of rats fed the basal diet. There were no significant differences in the liver lipid content for all groups; nor were changes observed in the protein content of the gastrocnemius muscle in the 3 groups fed the experimental diets as compared with that of the protein-free group.

Table 3 shows the changes in the concentration of threonine and amino acid N in plasma and liver observed in rats pair-fed the experimental diets.

The concentration of threonine in plasma was markedly lower in the imbalanced than in the basal group, although its consumption was the same for both groups. In contrast, the group fed the corrected diet showed a high concentration of threonine that could be correlated with the large intake of this amino acid. However, a high value was also observed in the group fed the protein-free diet in which there was obviously no threonine consumption. These results are similar to those observed in plasma histidine concentration in rats fed an imbalanced diet containing fibrin reported in a previous paper (4).

In sharp contrast with the results obtained in plasma, the liver threonine concentration was significantly higher in rats fed the imbalanced diet than in those on the basal diet. It is also noteworthy that this value for the imbalanced group was not significantly different from that observed in groups fed the corrected and the protein-free diets.

In plasma, the total amino acid N concentration was slightly higher in the group fed the imbalanced diet than in the other 2 groups. But in liver, the amino acid N percentage was substantially higher for groups fed the imbalanced and the corrected diets than for the basal group.

No differences were observed in plasma urea concentrations in groups fed the basal, the corrected, and the protein-free diet, but this value was substantially increased in the group fed the imbalanced diet.

These changes in threonine and total amino acid concentration observed in plasma and liver of rats fed the imbalanced diet are reflected in the values of the ratios derived. Some of the ratios were marked-

TABLE 3

Threonine, amino acid N and urea concentration in liver and plasma of protein depleted rats pair-fed the experimental diets for 24 hours [1]

Diet	Food intake	Threonine intake	Plasma [2]			Liver	
			Threonine	Amino acid N	Urea	Threonine	Amino acid N
	g/rat/24 hr	*mg/rat/24 hr*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 g*	*mg/100 g*
Basal A (10% wheat gluten) [3]	6.5 [4]	19.5	4.8	7.20	22.0	44.0 ± 2.2 [5]	49.2 ± 1.5
Imbalanced B (A + 3.1% AA mix.) [6]	6.5	19.5	2.3	8.50	49.0	60.9 ± 1.3 [7]	72.6 ± 2.6 [7]
Corrected C (B + 0.1% DL-threonine)	6.5 [4]	26.0	6.4	7.30	27.0	75.0 ± 11.2 [7]	64.0 ± 9.4 [8]
Protein-free	—	—	6.7	8.00	25.0	72.3 ± 6.5 [7]	68.8 ± 3.4 [7]

[1] Rats were killed 4 hours after the experimental period.
[2] From pooled blood of 6 rats.
[3] Supplemented with 0.8% of L-lysine as L-lysine·HCl, and 0.1% of DL-threonine.
[4] Pair-fed with the group fed the imbalanced diet B.
[5] Mean value ± SE of mean.
[6] For composition, see footnote 3, table 1.
[7] Highly significantly ($P \leq 0.01$) different from basal group mean.
[8] Probably significantly (P between 0.01 and 0.05) different from basal group mean.

TABLE 4

Relative concentrations of threonine and amino acid N in liver and plasma [1] of protein-depleted rats pair-fed the experimental diets for 24 hours [2]

Diet	PT/PAA [3]	LT/LAA [3]	LT/PT [3]	LAA/PAA [3]
	%	%		
Basal A (10% wheat gluten) [4]	7.7	10.4 ± 0.23 [5]	9.1	6.9
Imbalanced B (A + 3.1% AA mix) [6]	3.4	9.5 ± 0.43	26.5	8.5
Corrected C (B + 0.1% DL-threonine)	9.9	14.8 ± 0.81 [7]	11.7	8.5
Protein-free	7.0	10.8 ± 0.11	10.8	8.6

[1] From pooled blood of 6 rats.
[2] Rats were killed 4 hours after the experimental period.
[3] PT: threonine N in plasma; PAA: amino acid N in plasma; LT: threonine N in liver; LAA: amino acid N in liver.
[4] Supplemented with 0.8% of L-lysine as L-lysine·HCl, and 0.1% of DL-threonine.
[5] Mean value ± SE of mean.
[6] For composition, see footnote 3, table 1.
[7] Highly significantly ($P < 0.01$) different from basal group mean.

ly different from those observed in animals fed the more balanced diets. As shown in table 4, the ratio of plasma threonine to plasma amino acid N (PT/PAA), was markedly lower in the imbalanced rats than in animals fed the basal and the corrected diets. However, the liver threonine-to-plasma threonine (LT/PT) ratio was greatly increased for the imbalanced rats as the net result of the increase in liver with the concomitant decrease in plasma concentration. The values for these ratios in groups fed the basal and the corrected diets were similar to those for the protein-free group.

The liver amino acid N-to-plasma amino acid N ratio (LAA/PAA) was slightly increased in the imbalanced and corrected groups compared with the value for the basal group. No differences were observed in the ratio of liver threonine to liver amino acid N (LT/LAA) between the basal and the imbalanced groups, but this ratio was greatly increased in the corrected group.

DISCUSSION

Dietary amino acid pattern and organ composition. Of the changes observed in organ composition, those relating to the protein and amino acid content of liver are worthy of note.

Koeppe and Henderson (15) reported that an imbalanced diet may stimulate protein synthesis. Harper et al. (6) after isotopic experiments suggested that the excess of amino acids provided by an imbalanced diet could act by stimulating protein synthesis or by reducing the rate of protein breakdown.

The results of the present study, showing an increase in the threonine and amino acid N concentration in the liver of imbalanced rats that occurs simultaneously with the increase in the protein content, support the thesis that the stimulation of protein synthesis could be considered an initial effect of the imbalance.

It is conceivable that the liver of protein-depleted rats having an especially great need for protein takes up from the plasma the surplus of amino acids provided by the imbalanced diet for protein synthesis; but, as efficient synthesis occurs only when all essential amino acids are supplied in proper proportion, threonine, the limiting amino acid, would be drawn also from the plasma to a maximal extent to attain the right balance with the others. On this basis, a higher rate of liver protein synthesis in the group fed the imbalanced diet than in the group fed the basal one could be explained, even though the intake of threonine was the same for both groups.

However, as threonine is the deficient amino acid in the imbalanced diet, its concentration in plasma will fall to a level in which normal protein synthesis can no longer be supported.

This depression in plasma amino acid level is similar to that observed in previous studies of rats fed imbalanced diets (3–5) or of dogs after the consumption of a diet completely devoid in one essential amino acid (16).

The diminished total-body protein synthesis observed in long-term experiments (5) could be a consequence of the high threonine uptake which is reflected in the

below-normal plasma threonine concentration resulting from the concurrent depression in food intake.

These changes in the concentration of amino acids in plasma and liver lead to changes in the ratios that reflect the relative proportions in plasma and tissue fluids. Hence, the LT/PT and LAA/PAA ratios were higher in rats fed the imbalanced diet than in those fed the basal diet. The latter ratio was not so severely altered as the former, possibly owing to increased plasma amino acid concentration resulting from the imbalanced diet.

The decrease in plasma threonine concentration of imbalanced rats is reflected in the PT/PAA ratio that is lower for this group than for the group fed the basal diet.

The values for these ratios observed in animals fed the corrected diet are similar to those for the basal group, reflecting the more balanced pattern of amino acids provided by the diet. The values for the group fed the protein-free diet are also close to those of the basal group; this last result agrees with the observation made in a previous study (4) in which a balanced plasma amino acid pattern was produced after feeding a protein-free diet, despite the fact that this was accompanied by weight loss.

The results obtained in liver glycogen content were similar to those observed in long-term experiments (5). High levels of glycogen are associated with the consumption of diets containing the amino acid mixture, both in the ad libitum or in the pair-feeding experiments. These observations and those concerning liver lipids in which no changes were observed confirm the suggestion about the possible utilization of the excess of amino acids, not used for protein synthesis, for the synthesis of glycogen (5). However the fact that the glycogen was similarly increased in the liver of rats fed both imbalanced and corrected diets, does not support the thesis that the depression in food intake, characteristic of the imbalance, could be related to the high levels of liver glycogen.

The results obtained for gastrocnemius muscle weight and protein content in the pair-feeding experiments indicate that these values are not affected per se by the dietary amino acid pattern. Therefore the changes observed in ad libitum experiments would be only the consequence of the differences in food intake.

Appetite and body amino acid composition. The results of this study suggest that the depression in appetite could be related not only to an altered blood amino acid pattern, as reported (4), but also to changes in the relative proportion of amino acids between the plasma and tissue fluids. The depression in food intake in rats fed the imbalanced diet coincided with a high value for the LT/PT ratio as the result of the increase in liver and the decrease in plasma threonine concentration. In contrast, when the animals were fed the corrected diet and no signs of the imbalance occurred, the value for this ratio remained unchanged compared with that observed in rats fed the basal diet. The preference for a protein-free diet showed by rats that have been fed an imbalanced diet for a few days, reported in previous papers (2, 17), might also be explained as a consequence of the more normal distribution of amino acids between plasma and tissues; in fact, the value for the LT/PT ratio in protein-depleted rats was similar to that for the normal rat.

A similar picture was obtained when protein-depleted rats fed an imbalanced diet were injected with cortisol.[3]

Some of the results reported in this study are similar to others observed in kwashiorkor. Whitehead (18) and Stuart et al. (19) reported an altered blood amino acid pattern and high liver glycogen in children suffering from severe protein malnutrition. The theory that these symptoms could be related not only to a low protein intake or to a low protein-to-calorie ratio but also to the imbalanced amino acid pattern of the diets commonly used in areas in which kwashiorkor is prevalent (20) appears to be a reasonable working hypothesis.

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