An Eye Lesion in Rats Fed Low Chromium Diets [1]

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ABSTRACT In the course of experiments with rats raised with a 10% soy protein ration, the occurrence of a corneal opacity was observed. The present study was designed to determine whether this lesion is reproducible and whether it is related to the low chromium content of the diet (less than 100 ppb). Ten of 60 rats fed this ration without chromium supplement developed a pronounced opacity of the cornea and congestion of the iridal vessels. Supplementation of the drinking water with 2 ppm chromium prevented the appearance of the lesion, but did not cure the fully developed defect. The conjunctivae and the lens were not grossly affected.

The role of trivalent chromium in maintaining normal glucose tolerance has been demonstrated in man and laboratory animals (review (2)). More recent evidence established the eye as one of the several sites of action of the element. Isolated lenses from chormium-supplemented rats responded to insulin with higher glucose uptake than those from control animals (3, 4), but pronounced visible ocular changes have not hitherto been reported as a symptom of chromium deficiency.

In the course of experiments studying the effect of the element on various aspects of protein metabolism, rats maintained with rations low in protein and chromium developed a corneal lesion, grossly visible in one or both eyes. Chromium supplementation afforded full protection.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats (Walter Reed strain) were raised from weaning in groups of 10, 15, or 20. They were kept individually in plastic cages covered with perforated plastic boards. Sawdust (12 ppb chromium) was used as bedding material. The animals had free access to water and to a diet of the following percentage composition: Soy protein,[2] 10; distilled lard,[3] 8; sucrose, 77; Fox-Briggs salt mix (5), 4; and a vitamin mixture supplying in mg/kg diet: thiamine·HCl, 10; riboflavin, 10; pyridoxine, 5; vitamin B_{12}, 1; nicotinic acid, 40; folic acid, 2; biotin, 0.3; *p*-aminobenzoic acid, 100; Ca pantothenate, 50; inositol, 300; choline chloride, 2,500; menadione, 2; vitamin A acetate, 6; vitamin D_2 0.1; and *dl*-α-tocopheryl acetate, 500. The chromium content of this ration, as determined by atomic absorption spectroscopy (6) was consistently less than 100 ppb.[4]

In one experiment, described in table 1, vitamins A and D [5] were given by mouth dropper, in addition to the amount in the diet, supplying approximately an additional 0.75 mg of vitamin A and 12.5 μg of vitamin D per animal and week. The drinking water was triple-distilled and deionized, with a resistance approaching 18 megaohms/cm^2. In the latter experiments, it contained 0.1 ppm of molybdenum (as $Na_2MoO_4 \cdot 2H_2O$) and 1 ppm of vanadium (as $VOSO_4 \cdot 2H_2O$). The chromium-supplemented animals received 2 ppm of chromium (as $Cr(C_2H_3O_2)_3 \cdot H_2O$) in addition; in the earlier experiments 5 ppm was used (as $CrCl_3 \cdot 6H_2O$). The water and the diet, supplied in glass containers, were changed at least once a week. The animals were kept for a minimum of 10 weeks and were closely observed and weighed at weekly intervals. Eye changes were verified by use of an ophthalmoscope or a surgical lamp capable of light beam adjustment. Anesthesia was not required and the animals were held adequately quiet in the hands. All efforts were made to minimize contamination with metals.[6]

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[1] A preliminary report of these findings was given at the 7th International Congress of Nutrition (1).
[2] ADM Assay Protein C-1, Skidmore Enterprises, Cincinnati, Ohio.
[3] Distiller's Products, Inc., Rochester, New York.
[4] We thank Dr. F. J. Feldman for the chromium analyses.
[5] Oleovitamin A and D, N.F., The Vitarine Company, Inc., New York.
[6] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

TABLE 1

Incidence of corneal opacity in rats fed a low-protein ration

Beginning of experiment	Low-chromium rats		Chromium-supplemented rats	
	No.	No. with opacity	No.	No. with opacity
Jan. 1964	5	2	5	0
May 1965 [1]	15	2		
Aug. 1965	10	1	10	0
Nov. 1965	10	2	10	0
Feb. 1966	10	1	10	0
Apr. 1966	10	2	10	0
Total	60	10	45	0

[1] Group received additional supplement of vitamins A and D by mouth dropper.

RESULTS

The first visible indication of a pathological change occurred in some rats after they had received the diet for 2 to 3 weeks. Upon close inspection, the affected eyes of the rats fed low chromium had lost their natural brightness and the characteristic reflection of light was diminished. The symptom of this phase may remain unchanged during the experimental period, it may disappear spontaneously, or it may lead to the second phase after several weeks.

This stage consists of dilation of blood vessels in the iris of the affected eyes. It is not as readily reversible as the first phase and has disappeared only in one case. The congestion may remain the only symptom for the duration of the experiment, but in a significant number of cases (table 1) it developed into the third phase, after periods ranging from one to 8 weeks. This phase is characterized by the appearance of corneal opacity and subsequent neovascularization, in addition to the iridal congestion. The opacity is more frequently unilateral but is usually accompained by dilation of the iridal vessel in the other eye. Bilateral corneal lesions have been observed in 3 rats. The opacity begins as a small spot near the inner angle of the eye and increases in size until it eventually covers as much as half of the cornea. In these pronounced cases, a distorted shape of the pupil can be observed. The fully developed lesion is not reversible by chromium supplementation nor by feeding laboratory ration of normal protein content. The conjunctivae appear normal, and there is no grossly visible change of the lens (fig. 1).

The corneal opacity was observed in 6 of 6 consecutive experiments, from January, 1964, to August, 1966. It was present in 10 of 60 rats fed low chromium, whereas none of 45 chromium-supplemented animals showed any changes not even those of the first phase. The lesion was not prevented by additional supplements of vitamins A and D (table 1).[7]

DISCUSSION

That no visible ocular changes were detected in any of the chromium-supplemented rats implicates the low chromium state as the main cause of the corneal opacity. The suboptimal protein content of the diet may have been a contributing factor, since eye lesions have been observed only occasionally in rats raised with a 30% Torula yeast ration, which is also of a low chromium content (less than 100 ppb). The absence of any conjunctival changes, as well as the ineffectiveness of additional vitamin A supplements rules out deficiency of this vitamin as a major cause. External trauma to the cornea as the cause is unlikely, because internal changes in the eye precede the development of the corneal opacity. The lesion is not related to overt diabetes, since the afflicted rats are not glycosuric; however, a mild impairment of glucose tolerance does exist in rats fed low chromium.

The sequence of events and the biochemical mechanism by which the low chromium state leads to this pathological change remains to be elucidated by ophthalmological and micropathological studies, now in progress. The question is of interest whether a similar lesion, caused

[7] After completion of the experiments reported in table 1, the diet-making facilities of this Institute were closed and the diet was prepared to specifications by a commercial organization (General Biochemicals, Chagrin Falls, Ohio). The chromium content of different batches ranged from 190 to 350 ppb, and no opacities developed in 2 groups of rats kept for 12 and 6 weeks, respectively. Subsequently, a batch of diet was prepared by the authors, with a chromium content of less than 100 ppb. Feeding this ration resulted in the appearance of the corneal opacity in one of ten low chromium rats, after 7 weeks. The chromium-supplemented animals were normal. The high chromium content of the commercial diet was traced to the sucrose used and another batch of sucrose, low in chromium, was substituted. The new diet lots had a chromium content of from 75 to 82 ppb and produced the opacity in one of 10 rats after 6 weeks. The chromium-supplemented rats were again fully protected.

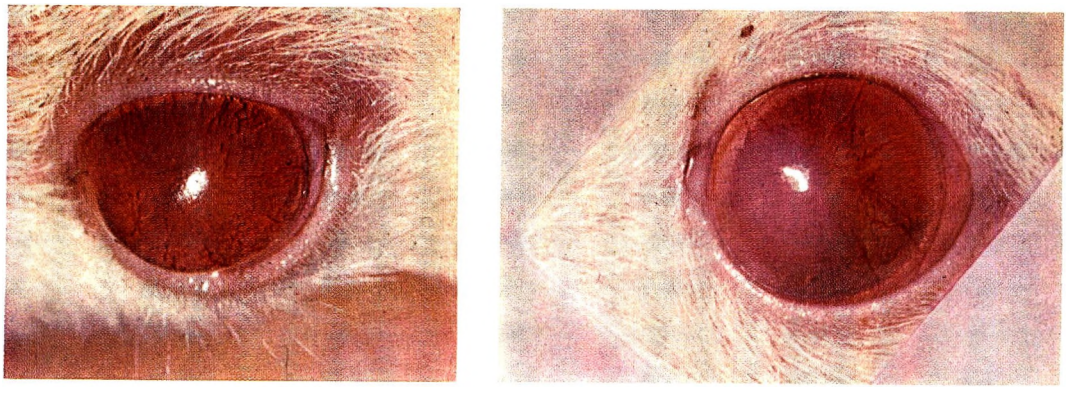

Fig. 1 Photographs showing both eyes of a rat in vivo, with congestion of the iridal vessels in both eyes, and corneal opacity in eye to the right. The small bright spots are reflections of the light used and are not related to the lesion. (Photographs courtesy of Dr. John A. Buesseler, Columbia, Missouri.)

by a combination of low protein and low chromium states, exists in other species, particularly in man. That protein deficiency in man can be complicated by chromium-responsive metabolic disturbances has been demonstrated (7).[8]

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Effect of 6-Aminonicotinamide and of Added Vitamin A on Fusion of Embryonic Rat Palates in vitro [1]

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ABSTRACT A procedure for the in vitro cultivation of embryonic rat palatal tissue, using semi-defined media, is described. Palatal fusion obtained in vitro resembles that normally seen in vivo. The effect of 6-aminonicotinamide (6 AN) a niacin inhibitor and of excess vitamin A on palatal fusion was investigated using this in vitro system. Administration of 6 AN to the mother resulted in a high incidence of failure to fuse and of partial fusion. This compound appeared to produce clefts by interfering with the ability of the shelves to fuse but not otherwise affecting their development. This effect was prevented by the simultaneous administration of nicotinamide to the mother. Addition of 6 AN to the culture medium was less effective and was not reversed by addition of nicotinamide to the medium. Histological changes were seen in most treated palates regardless of the route of administration of 6 AN. These changes could be prevented by nicotinamide, either injected or added to the culture medium, but no relation could be found between the tissue changes and the occurrence of clefts. Addition of vitamin A to the culture medium resulted in a high incidence of failures of palates to fuse. This appeared to be due to retarded development of the shelves, with failure to meet in the midline. Experiments with sliced palatal preparations indicated that the tissues were competent to fuse if they were able to come together. Alcian Blue staining and autoradiography with $^{35}SO_4$ did not indicate that mucopolysaccharide synthesis or distribution or sulfur metabolism are factors involved in the vitamin A effect. Evidence is presented supporting the view that palatal fusion may be interrupted by nutritional factors operating through different mechanisms, although the end result observed at birth (cleft palate) is the same.

In a previous publication (1)[4] techniques were described for the cultivation of embryonic rat palates in vitro on semi-defined media. Under these conditions the embryonic palatal shelves fuse in the midline, with the disappearance of certain epithelial elements and merging of the underlying mesenchymal tissue at the fusion line. Moriarty et al. (2), Konegni et al. (3), and Pourtois (4) have described similar techniques. These procedures lend themselves to a study of the effects of nutritional factors on the formation of developmental anomalies.

Several nutritional deficiencies, including those of riboflavin, folic acid, and niacin, when induced in pregnant rats, result in cleft palate in the offspring at term (5–7). Chamberlain and Nelson (8) and Chamberlain (9) administered 6-aminonicotinamide (6 AN), a niacin antagonist, to rats as late as day 15 of gestation and observed cleft palates in the majority of the embryos on days 17 to 19. This effect was abolished if niacin was given at approximately the same time as the 6 AN.

Hypervitaminosis A has also been shown to interfere with normal palatal closure and fusion, as demonstrated by the formation of cleft palates in the offspring of treated mothers (10, 11). It is not known whether the excess vitamin A influences the competency of the palatal shelves to fuse along their medial margins or whether it influences other aspects of head

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[4] A manuscript with a detailed description of the technique is in preparation.

development which may interfere with the proper apposition of the shelves before fusion.

In this paper we will describe the effect of 6 AN on the fusion of embryonic rat palates when the antagonist is given to the mother and the palatal tissue is cultured in vitro and when 6 AN is added directly to the culture medium. Evidence will be presented indicating that 6 AN and excess vitamin A act by different mechanisms, although the gross result (cleft palate) is the same.

EXPERIMENTAL

Culture methods. Details of the procedures for mating animals, for estimating embryonic age, and for the removal, preparation and culturing of embryonic rat palates have been presented elsewhere (1).[5] Briefly, rats of the Sprague-Dawley strain were mated overnight and the morning on which spermatazoa were found in the vaginal smear was designated as day zero of gestation. Animals were maintained with commercial rat pellets, with water and food ad libitum. On day 14+ or 15+ the embryos were removed aseptically and the middle one-third of the head, containing the palatal shelves and associated structures, was dissected free of the other parts of the head. The tissue was cultured according to a modification of the method of Trowell (12). The culture medium consisted of 80% Leibovitz medium L-15 (13)[6] and 20% fetal calf serum. After 24 hours in culture at 37° the medium was renewed. Sterile technique was used throughout. After a total of 72 hours in culture the tissues were fixed in Bouin's solution, embedded in paraffin, serially sectioned at 10 μ, and stained with Harris hematoxylin and eosin except as otherwise noted. Some preparations were stained with Alcian blue according to the procedure of Pearse (14) in order to visualize the distribution of mucopolysaccharides in the palatal shelves.

Administration of 6 AN and of vitamin A. When administered to the mother, 6 AN[7] was given in a single intraperitoneal injection as an aqueous solution (8 mg/kg body weight) on day 12+ of gestation. In preliminary experiments to check the efficacy of 6 AN in causing palatal clefts the embryos were removed on day 19 and examined grossly. When incorporated in the culture medium 6 AN was added to give a final concentration of 0.05 to 0.5 mg/ml. Nicotinamide was given intraperitoneally at the same level (8 mg/kg) as 6 AN or was incorporated in the culture medium to give a final concentration of 0.05 to 0.5 mg/ml.

Vitamin A was added to the medium to yield a concentration of either 25 or 50 IU/ml. Aquasol A,[8] an aqueous preparation of natural vitamin A, was used. The vehicle used in the manufacture of Aquasol A was supplied to us by the manufacturer and was incorporated in the control medium in volumes equal to those added with the vitamin A.

To examine the direct effect of 6 AN on palatal fusion, newly dissected embryonic palates were placed in Tyrode's solution or in Tyrode's solution containing 1 mg 6 AN/ml for periods of 10 to 30 minutes. They were then washed and were placed directly in the culture chambers on medium which did not contain 6 AN. They were cultured and handled as described.

Radioautography. $Na_2{}^{35}SO_4$ (carrier-free) was added to culture media with or without added vitamin A to give a final concentration of either 0.1 μCi/ml or 0.5 μCi/ml and palates were cultured as described. The fixed palates, serially sectioned at 10 μ and mounted on slides, were dipped in Eastman Kodak NTB 2 Nuclear Track Emulsion[9] and exposed at 5° for 3 weeks. They were then developed in an Amidol developer and stained with Mayer's hematoxylin and eosin.

Slice experiments. To investigate the *competency* for fusion on the part of vitamin A-treated palates, some preparations were sliced longitudinally in the midline, between the 2 shelves. The halves were then placed on rayon acetate squares and moved together until the medial edges of the shelves were in contact. They were cultured on media with or without added

[5] See footnote 4.
[6] This synthetic medium contains 1 mg nicotinamide/ml. It does not contain vitamin A.
[7] K and K Laboratories, Hollywood, California.
[8] U. S. Vitamin and Pharmaceutical Corporation, New York.
[9] Nuclear Science and Engineering, Pittsburgh.

vitamin A and prepared for histological examination as described.

RESULTS

Figure 1 shows a control (untreated) palate cultured for 72 hours on L-15 medium with 20% fetal calf serum. The area of fusion is indicated by arrows. Cultures were recorded as "fused" when the fusion extended over more than two-thirds of the length of the palate and as "partially fused" when the fusion extended over more than one-third but less than two-thirds of the length of the palate. Otherwise they were recorded as "not fused."

When 6 AN (8 mg/kg body weight) was given to the mothers on day 12+ of pregnancy, seven of seven embryos had completely cleft palates on day 18. This established the conditions and dosage for subsequent experiments with this antimetabolite. Table 1 shows the effects on palatal fusion of administering 6 AN or 6 AN plus nicotinamide on day 12+ of pregnancy and culturing the palatal tissue in vitro on day 14+ or 15+. Of 27 control palates, 21 were fused. When 6 AN was given 19 of 53 palates were not fused and 20 were only partially fused. This effect was prevented by administering nicotinamide (8 mg/kg) immediately following the 6 AN injection. Figure 2 shows a palate from an embryo of a 6 AN-treated mother. The shelves are in close contact and the medial edges are flattened and thickened, suggesting that the shelves are pressing against one another. There is no evidence of fusion; the 2 layers of epithelium are adjacent to one another but both are still intact and there is no sign of mesenchymal invasion at the point of contact. On the oral aspect of the palatal shelves there is frequently seen a clear space or band between the epithelium and the mesenchyme. This is not identical with the basement lamina. It is not clear whether this is a hyaline material or a

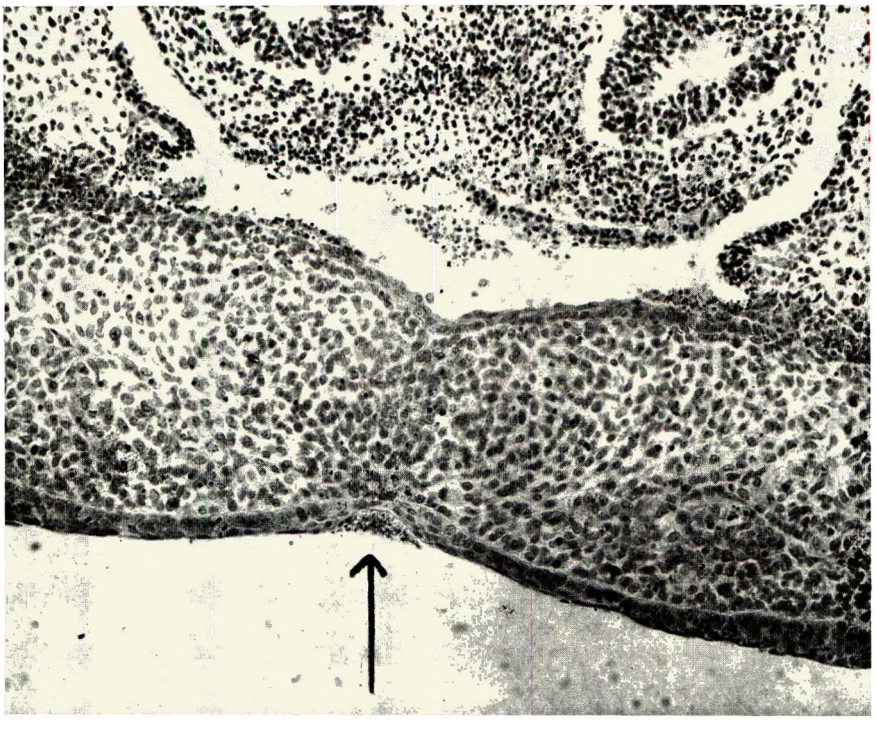

Fig. 1 Transverse section of a rat palate from a 14+ day normal embryo. Cultured 72 hours on Leibovitz L-15 medium containing 20% fetal calf serum. Midline fusion indicated by arrows; 10 μ. H & E. $\times$ 128.

TABLE 1

Fusion of embryonic Sprague-Dawley rat palates in vitro following administration of 6-aminonicotinamide (6 AN) or of 6 AN and nicotinamide to the mother or of 6 AN to the mother and nicotinamide to the palatal tissue [1]

Treatment		Palatal fusion		
Mother	Palatal tissue	Fused	Partially fused	Not fused
None	none (control)	21(78%)	4(15%)	2(7%)
6 AN	none	14(26%)	20(38%)	19(36%)
6 AN + nicotinamide	none	9(69%)	3(23%)	1(8%)
6 AN	nicotinamide	5(28%)	8(44%)	5(28%)

[1] The 6 AN and nicotinamide were administered to the mother intraperitoneally at levels of 8 mg/kg body weight, each, on day 12+. Nicotinamide was incorporated in the culture medium at 0.1 mg/ml. Palates were removed for culture on day 14+, and were cultured for 72 hours.

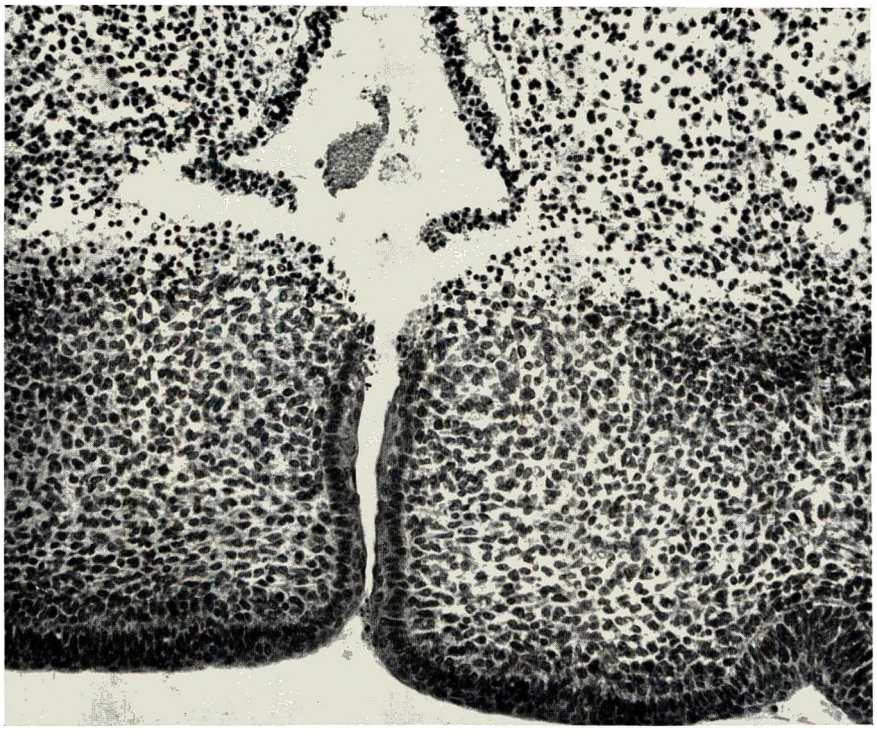

Fig. 2 Transverse section of a 14+ day rat palate from embryo of a mother given 6-aminonicotinamide (8 mg/kg body weight) on day 12+ of gestation. Cultured 72 hours on same medium as palate in figure 1. Note lack of fusion of palatal shelves, but with apposition of medial margins; 10 μ. H & E. $\times$ 128.

space, but it is never seen in untreated preparations. Frequently the epithelial layer contains pyknotic nuclei and less well-defined cell outlines. These changes are most marked in palates on removal from the uterus and they become less prominent during the time that the palates are cultured, whether on control media or on media to which nicotinamide has been added. Anterior to the palatal shelves, in the region of the primary palate, other histological changes are seen in treated embryos but not in control embryos. These are shown in figures 3 and 4. In the treated embryos the areas indicated by arrows appear to have pyknotic nuclei and vacuolated cytoplasm. The impression of cellular organization, seen in the controls, is lack-

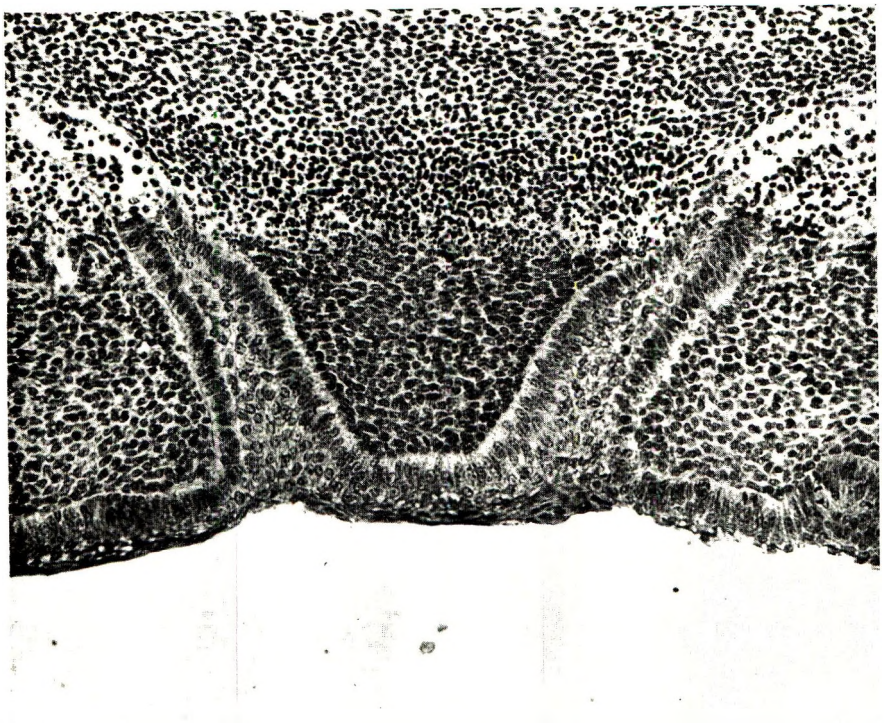

Fig. 3 Transverse section through region of the primary palate of a control embryonic rat palate preparation. Cultured for 72 hours on control medium. H & E. × 128.

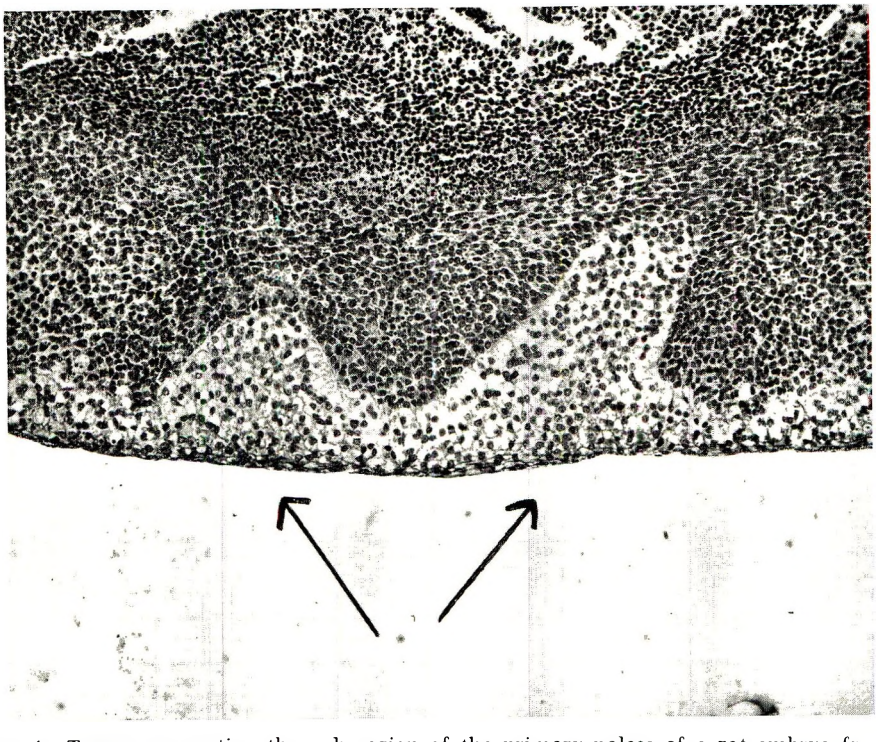

Fig. 4 Transverse section through region of the primary palate of a rat embryo from a mother given 6-aminonicotinamide (8 mg/kg body weight) on day 12+ of gestation. Note vacuolated cytoplasm and lack of cellular organization in areas marked with arrows. H & E. × 128.

ing in the treated embryos. The clear space or band, described above, is not seen in the region of the primary palate but occurs more posteriorly on the shelves. Both types of histological changes are very marked in sections of uncultured palates from treated embryos and become progressively less striking as the palates are cultured in vitro. When nicotinamide is given to the mother at the same time as 6 AN these histological changes do not appear.

When 6 AN was given to the mother and nicotinamide was incorporated in the culture medium 72% of the palates were either not fused or were partially fused. This is similar to the results with 6 AN alone (74%) and indicates that nicotinamide had no effect on palatal fusion under these conditions.

The effects of adding 6 AN to the culture medium is shown in table 2. At levels of 0.1 and 0.5 mg/ml 6 AN appears to have an effect, in that the proportion of partial fusions is increased, but the effect is much less striking than when 6 AN is given to the mother. Nicotinamide was not able to reverse the 6 AN effect when added to the medium in the same concentration as 6 AN, although it was able to do so when given to the mother at the same time as 6 AN. However, this may be due to the fact that nicotinamide alone appears to interfere with fusion when present in high enough concentration. This is in agreement with the conclusion of Smithberg (15) concerning the incidence of congential malformation in mice treated with nicotinamide.

To limit the time of exposure of palatal tissue to 6 AN, palates were removed from untreated embryos and placed in Tyrode's solution containing 1.0 mg/ml of 6 AN. After 10–30 minutes the tissue was removed and cultured on medium without 6 AN. Under these conditions fusion occurred in all of the palates cultured, but the histological changes described above were observed.

Figure 5 shows a cross section of a palate cultured on medium containing 25 IU/ml of vitamin A. The palatal shelves are clearly separated and the epithelial surfaces are intact. Palates cultured on medium containing only the diluent fused and were histologically indistinguishable from control palates such as that shown in figure 1. Table 3 shows the number of control and vitamin A-treated palates cultured and the number of each that showed complete or partial fusion. It is clear that vitamin A, at 25 to 50 IU/ml, interferes with palatal fusion in vitro.

When sections were stained with Alcian Blue no gross differences were observed between control and vitamin A-treated palates. Mesenchymal elements stained pale blue, but there were no differences in the distribution or intensity of staining when control (fused) palates were compared with palates exposed to 25 or 50 IU vitamin A/ml (not fused).

No gross differences were observed in the density or distribution of exposed

TABLE 2

Fusion of embryonic Sprague-Dawley palates in vitro in media containing 6-aminonicotinamide (6 AN), 6 AN and nicotinamide, or nicotinamide [1]

Treatment	Palatal fusion		
	Fused	Partially fused	Not fused
None (controls)	21(78%)	4(15%)	2(7%)
0.05 mg/ml 6 AN	5(100%)	0	0
0.10 mg/ml 6 AN	9(45%)	9(45%)	2(10%)
0.50 mg/ml 6 AN	0	2(100%)	0
0.1 mg/ml 6 AN + 0.1 mg/ml nicotinamide	8(28%)	5(72%)	0
0.1 mg/ml nicotinamide	2(40%)	2(40%)	1(20%)

[1] Palates were cultured for 72 hours, beginning on day 14+ of gestation.

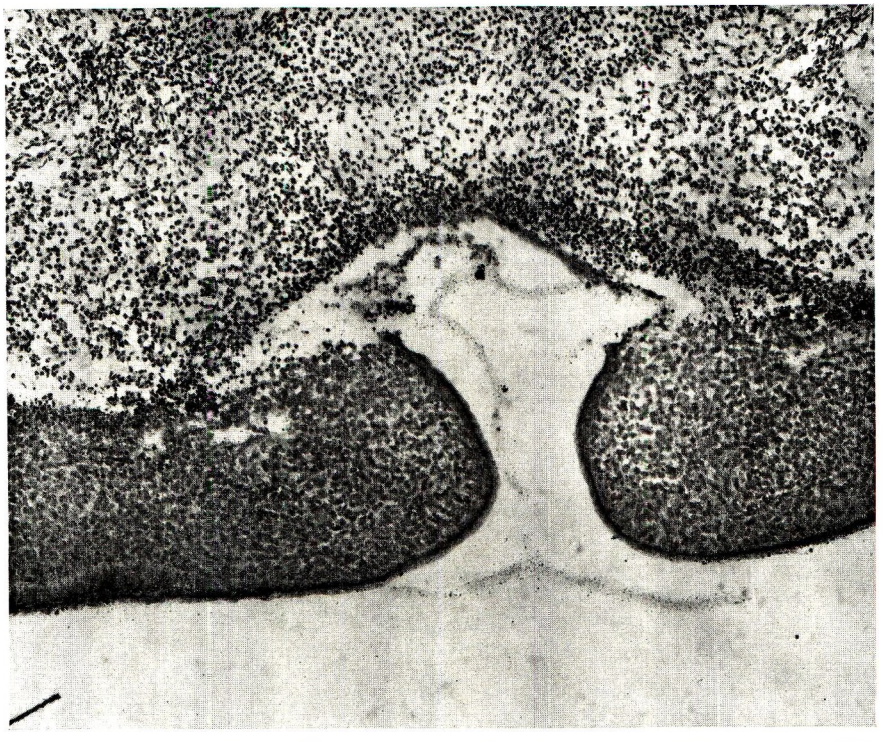

Fig. 5 Transverse section of 14+ day embryonic rat palate cultured in vitro for 72 hours on medium containing 25 IU aqueous vitamin A/ml. Note lack of apposition and fusion of palatal shelves. H & E. × 128.

TABLE 3

In vitro fusion of control and vitamin A [1]-treated embryonic Sprague-Dawley rat palates

Treatment	Total no. palates	No. not fused	No. fused
Control [2]	10	1(10%)	9(90%)
Vitamin A [3]	29	25(86%)	4(14%)

[1] Aquasol A, National Vitamin Corporation.
[2] Leibovitz L-15 medium with 20% fetal calf serum.
[3] Same medium with 25 IU vitamin A/ml.

silver grains in radioautographs of control palates as compared with palates cultured in media containing either 25 or 50 IU of vitamin A /ml. Areas of developing cartilage showed heavy concentrations of ^{35}S in both control and vitamin A-treated palates but the palatal shelves exhibited much less ^{35}S in both preparations. In the shelves, ^{35}S was taken up by the mesenchymal elements but no accumulation was visible in the epithelial surfaces.

Figure 6 is a photograph of a palatal preparation which had been sliced longitudinally, moved together until the medial edges of the shelves were contiguous, and cultured on L-15 with 20% fetal calf serum. Figure 7 shows a similarly treated preparation cultured on the same medium to which had been added 25 IU vitamin A/ml. It is seen that both palates exhibit midline fusion with disappearance of epithelial elements.

DISCUSSION

Before discussing the experimental data, it is desirable to consider the in vitro cultivation techniques used, with respect to their limitations and to their advantages. As the palatal tissue cannot readily be isolated earlier than 14+ days, it is not possible to apply nutrients and antagonists earlier than approximately 18 to 24 hours before fusion normally would occur. In many in vivo teratologic studies, the mothers are given the compound earlier than 14+ days and it is difficult to compare these studies with in vitro studies as described here. The removal of a large

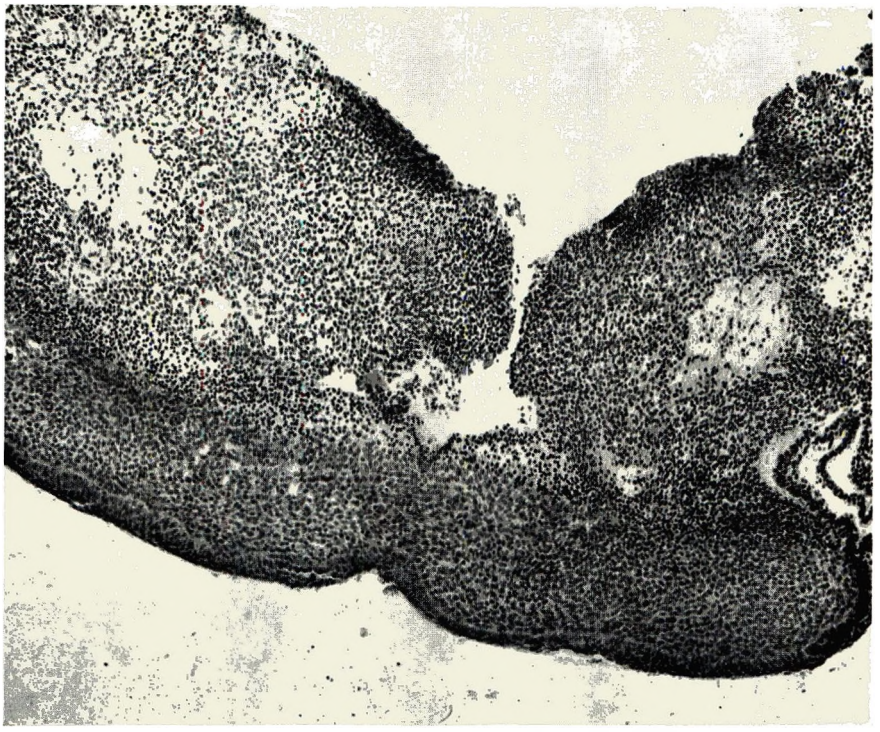

Fig. 6 Transverse section of a 14+ day embryonic rat palatal preparation, sliced longitudinally moved together until the medial edges of the shelves were contiguous, and cultured 72 hours on control medium. H & E. × 128.

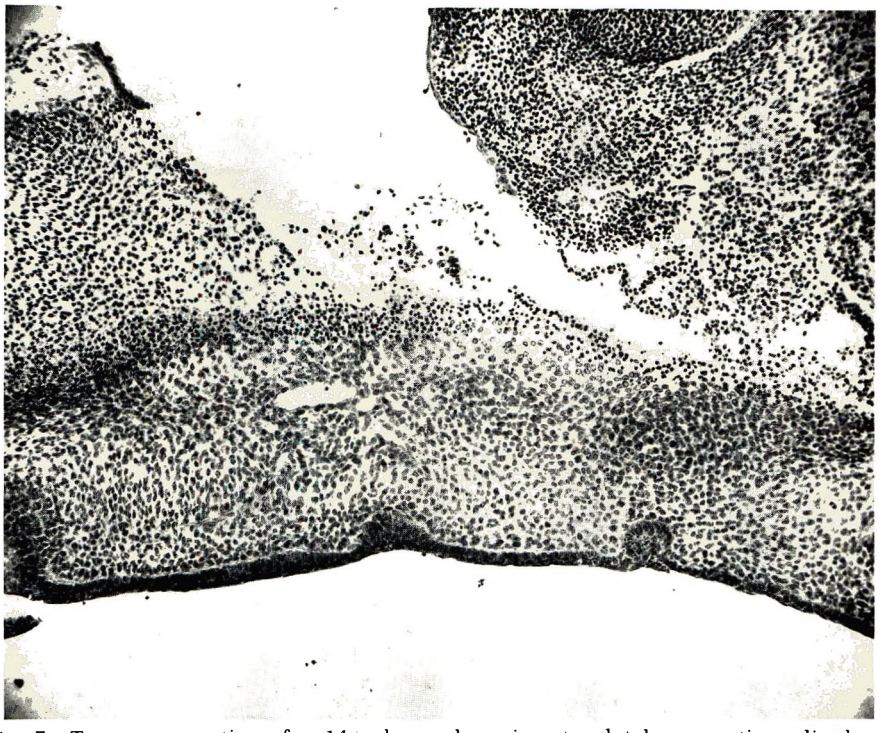

Fig. 7 Transverse section of a 14+ day embryonic rat palatal preparation, sliced as in figure 6, and cultured for 72 hours on medium containing 25 IU vitamin A/ml. H & E. × 128.

part of the head, as well as the tongue and mandible, disrupts the normal relationships between palatal development, overall growth of the head, and movement of the tongue. On the other hand, this has the advantage that general head growth and tongue movement do not obscure the direct effect of added compounds on palatal tissue. Finally, the in vitro techniques circumvent the problems of placental transfer of the compound of interest and the question of whether the compound is transformed to an "active" material by the mothers' tissues before exerting its effect on the embryo.

Although, for reasons given above, it can not be assumed a priori that in vitro studies of palatal fusion will be in agreement with data obtained from in vivo experiments, the results reported here are in good agreement with the data of Chamberlain and Nelson (8). The niacin antagonist, 6-aminonicotinamide, interferes with palatal fusion when given to pregnant rats and the changes induced are not corrected by removing the tissue from the maternal environment and culturing it on a normal medium. The tissues were removed from the maternal environment approximately 24 hours before they would be expected to fuse, but the changes persisted.

Incorporation of the vitamin antagonist in the medium was much less effective, although Chamberlain and Nelson showed that giving it to the mother on day 15 of gestation still caused cleft palate. One can not compare effective dosages delivered to the tissues by the 2 methods and it is not known whether there is a metabolic alteration of the antagonist, either in the mother or in the embryo, so the difference in activity in vivo and in vitro can not be explained. The fact that nicotinamide administration to the mother can reverse the 6 AN effect even when the palates are removed and cultured in vitro supports the view that 6 AN is acting as a competitive inhibitor of nicotinamide. Nicotinamide is not able to do this when the 2 compounds are added to the culture medium but this may be because the total concentration is so high that both compounds are acting as teratogens.

The histological changes are of interest because they appear to be a consequence of 6 AN activity. However, it does not seem that these changes represent the alteration which prevents fusion of the palates, as it is possible to separate the 2 phenomena. The addition of nicotinamide to medium containing 6 AN abolishes the histological changes, but fusion still does not occur. However, dipping the tissue in Tyrode's solution containing 6 AN produces the histological changes without interfering with fusion. Therefore, it appears that the 2 effects are separate and distinct consequences of 6 AN activity.

Whether 6 AN is given to the mother or incorporated in the medium, the shelves grow and come in contact with one another. However, changes which should occur at the point of contact, with epithelial dispersion or degeneration, do not take place. This suggests that 6 AN has exerted some effect on the tissues so that the shelf margins are no longer competent to fuse.

The results reported here also indicate that added vitamin A acts directly on the palatal tissues to interfere with normal fusion. There are no obvious histological changes that would account for the failure of these palates to fuse, but in agreement with the observations of Holdsworth (16) the palatal shelves of treated cultures appear smaller and less well developed than do palatal shelves of control cultures. Although vitamin A function is frequently interpreted in terms of development of cartilaginous structures (17, 18), it should be noted that the palatal shelves contain no cartilage at this stage of development. The slice experiments indicate that the medial margins of vitamin A-treated palatal shelves retain the ability to fuse if they come in contact with one another and that excess vitamin A does not alter the shelf surfaces. The region of fusion competency appears to be limited only to these medial margins, since when the slices are placed together so that the medial margin of one shelf contacts a different surface of the other shelf no fusion occurs, either in control or vitamin A-treated cultures. This bears analogy with the specific tissue-

tissue interaction recognized in the development of the lens (19) but has not been reported, to the best of our knowledge, in palatal development. It may represent a special morphogenetic competency characteristic of only that part of the palatal surface on the medial edge of the shelf.

The studies of Larsson (20) on palatal development in mice, using several histological stains and radioautography of embryos from mothers given $^{35}SO_4$ pointed to a role of sulfomucopolysaccharides in palatal closure but not necessarily in palatal fusion. Other workers [10] have observed a sharp increase in $^{35}SO_4$ incorporation into palatal tissue approximately 48 hours before closure and a marked decrease in $^{35}SO_4$ incorporation shortly after closure. This has been interpreted as indicating increased sulfomucopolysaccharide synthesis before closure, which is in agreement with the findings of Larsson. Our studies with Alcian Blue staining and with $^{35}SO_4$ autoradiography do not indicate a role of sulfomucopolysaccharides in palatal fusion, nor do they indicate that vitamin A influences mucopolysaccharide concentrations or synthesis under the conditions used here. As pointed out earlier, in the in vitro system $^{35}SO_4$ cannot be added to the medium before 14+ days and for this reason the increase in mucopolysaccharides observed by Larsson would not have been seen.

On the basis of these data it is suggested that excess vitamin A retards development of the palatal shelves so that they do not come in contact with one another at the proper time. Head growth subsequently moves them further apart so that fusion can not occur. The tissue competency is not affected. On the other hand, 6 AN affects the competency of the tissues to fuse and although the shelves come together they do not fuse and subsequent head growth pulls them apart, leaving a cleft. Therefore, the 2 agents (excess vitamin A and 6 AN) produce the same end result (cleft palate) but by 2 different mechanisms. Studies in which embryos are examined after day 17 or 18 will show only the end result (cleft palate) and differences in mechanism of action of agents will not be recognized.

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Morphological and Respiratory Changes in Rat Liver Mitochondria Resulting from a Low Casein Diet

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ABSTRACT The influence of a low casein diet on rat liver mitochondria was studied. The low casein diet contained 4% casein, and the control diet, 25%. Rats were fed the test diets for 40 to 50 days, at which time their livers were homogenized and the mitochondria isolated. Electron photomicrographs of isolated mitochondria from rats fed the low casein diet showed swelling and irregular shape. Simultaneously, using succinate as the substrate, oxygen consumption was measured polarographically with an oxygen electrode. Oxygen consumption in the presence of ADP and succinate was greater in the rats fed the low casein diet than in those fed the high casein diet. The respiratory control index was also greater in rats fed the low casein diet. These studies indicate that phosphorylative activity of mitochondria was not impaired, but rather, was activated. In another experiment the influence of caloric intake was examined. In rats fed a restricted amount of the 25% casein diet, no mitochondrial change was observed. It was concluded that the mitochondrial change observed in rats fed the low casein diet was not due to low food intake.

A number of investigators have reported liver injury to be induced when rats are fed a low casein diet (1–5). The studies reported have concerned the relationship between this phenomenon and deficiencies of sulfur-containing amino acids and vitamin E in the diet (1–9).

To investigate this phenomenon biochemically and physiologically, it must be studied not only by using tissue slices or whole homogenate, but also by using subcellular particles such as mitochondria. Moreover, since energy efficiency is less with a decreased dietary casein level (10), the use of mitochondria appears to be important in carrying out the study. Mitochondrial variations under conditions of malnutrition, such as vitamin B_2 deficiency (11), essential fatty acid deficiency (12–17) and diabetes (18), have been reported.

In those previous experiments using mitochondria, the measurement of oxygen consumption was made by the Warburg manometric method in most cases. But to measure the oxygen uptake by intact mitochondria, the use of an oxygen electrode seemed appropriate because incubation can be carried out without shaking, and measurement of oxygen consumption can be made during short time-periods. Also previous incubation of mitochondria is not necessary. Therefore, the oxygen electrode was used for measurement of oxygen consumption in the experiments reported here.

The results showed that feeding rats a low casein diet resulted in swelling of liver mitochondria and an increase in oxygen consumption and the respiratory control index.

EXPERIMENTAL

Animals and diets. Male rats of the Donryu strain, weighing from 100 to 170 g, were used. They were housed in individual cages in a temperature-controlled room at about 22°.

The composition of the diets is shown in table 1. The high and low casein diets contained 25 and 4% casein, respectively. Both diets contained 5% corn oil, 5% salt mixture, 0.25% vitamin mixture and 0.15% choline chloride and were made up to 100% with potato α-starch. The salt and vitamin mixtures were made according to Harper (19). But the concentration of each vitamin in the diets was one-third that used by Harper, except in experiment 3, as described below.

Preparation of mitochondria. Mitochondria were prepared according to Hagihara (20) and Chance and Hagihara (21, 22).

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TABLE 1
Composition of diets [1,2,3]

	Low casein diet	High casein diet
	%	%
Casein	4	25
α-Starch [4]	85.6	64.6
Corn oil	5	5
Mineral mixture B (19)	5	5
Vitamin mixture (19)	0.25	0.25
Choline chloride	0.15	0.15

[1] Diets were supplemented further with 6,000 IU of vitamin A and 600 IU of vitamin D/kg of diet.
[2] In experiment 3, two 4% casein diets were used, to one of them was added the amount of vitamin mixture indicated in this table, and to the other was added threefold this amount. To the latter diet 100 mg vitamin E/kg of diet were further added.
[3] 1.74 g of phosphorus/kg of diet was added to two 4% casein diets in experiment 3 to make the phosphorus content the same as that of the 25% casein diet.
[4] Sweet potato α-starch, obtained from Terahiko Seifun Company, Japan.

The preparation medium contained 0.21 M mannitol, 0.07 M sucrose, 0.1 mM EDTA, and 0.01 M Tris buffer at *p*H 7.4. When bovine serum albumin was added, its final concentration in the medium was 0.05%.

After the rats were killed, the livers were removed quickly, and placed immediately into 4 volumes of ice-cold preparation medium. It was homogenized with a Teflon homogenizer and cooled at 0°. The homogenate was centrifuged at 600 × *g* for 5 minutes to sediment nuclei and cell debris; then the supernatant fraction was centrifuged at 10,000 × *g* for 8 minutes to sediment mitochondria. This mitochondrial fraction was again suspended in the preparation medium, and the suspension was centrifuged at 600 × *g* for 3 minutes to remove the nuclear contamination. The supernatant fraction then obtained was again centrifuged at 10,000 × *g* for 8 minutes, and the sedimented fraction was used as the mitochondrial fraction for measurement of oxidative activity. For the electron photomicrographs,[1] in somes cases, the mitochondrial fraction was further purified by washing repeatedly with the preparation medium.

Assay of oxidative activity using succinate as substrate. Oxygen consumption and the respiratory control index using succinate as the substrate were measured polarographically by the method of Hagihara (23), using the oxygen electrode. The composition of the reaction medium described by Hagihara was modified slightly.

The reaction medium contained 0.28 M mannitol, 0.01 M potassium phosphate buffer at *p*H 7.4, 0.25 mM EDTA, 0.01 M Tris buffer at *p*H 7.4, 0.01 M KCl and 0.005 M $MgCl_2$.

The mitochondrial suspension was made by suspending the mitochondrial fraction in 0.5 ml of preparation medium/g of tissue used. The protein concentration of mitochondrial suspension was measured by the biuret reaction (24) or by a semimicro-Kjeldahl method. Incubation was carried out at a temperature of 30°.

The oxygen electrode coated with collodion membrane and the closed reaction cell were used. Oxygen consumption was indicated directly on the recorder. Initial oxygen concentration in the reaction mixture was assumed to be the same as that in pure water at 30°.

A typical curve of oxygen consumption obtained with the oxygen electrode is shown in figure 1. First, 3.4 ml [2] of reaction medium were put into the reaction cell, and after the recorder pen stabilized, 0.2 ml of mitochondrial suspension was added. Here, oxygen consumption at stage 1 was recorded. Then, as the substrate, succinate solution was added in an amount to make the final concentration in the reaction cell 7 mM. Oxygen consumption at stage 2 was recorded. Next, ADP solution was added in an amount to make the final concentration in the reaction cell 146 μM. Oxygen consumption increased and was recorded as stage 3. When the added ADP was consumed, the oxygen consumption rate decreased, and this stage was recorded as stage 4.

In these experiments, oxygen consumption per milligram of protein at stages 3 and 4 was measured, and the respiratory control index was calculated.

Electron photomicrography of isolated mitochondria. Mitochondria were prepared by the same method as that when oxygen consumption was measured. The

[1] The electron photomicrograph of the mitochondrial fraction obtained after washing the mitochondria once with the preparation medium showed a little contamination from the microsome fraction, and hence the mitochondria were purified by washing repeatedly with the preparation medium to remove microsome contamination.
[2] Volume of the reaction cell was 3.7 ml.

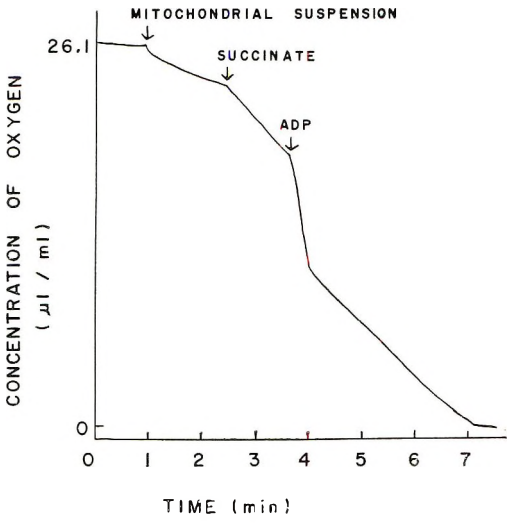

Fig. 1 Typical curve of oxygen consumption measured polarographically using mitochondria from a rat fed the 4% casein diet. The arrows indicate where mitochondrial suspension, succinate and ADP were added to the incubation medium. Mitochondrial suspension was added first, and after incubation, succinate was added; following further incubation, ADP was added.

electron photomicrographs were taken according to Burgos et al. (25).

Plan of experiments. The experiments were carried out in 3 phases as experiments 1, 2 and 3.

In experiment 1, the rats were divided into 2 groups, one group was fed a high casein diet, and the other a low casein diet. Both groups were fed the test diets ad libitum for 40 to 50 days. Then, the rats were killed, the liver mitochondria isolated, and the oxygen consumption was measured. Electron photomicrographs were taken of several samples.

In experiment 2, the effect of caloric restriction of rats fed the high casein diet on oxygen consumption was examined. In this experiment, rats were divided into 3 groups: group 1, fed 25% casein diet, ad libitum; group 2, fed 4% casein diet ad libitum; and group 3, fed 25% casein diet, restricted. The restricted 25% casein diet was given to the rats of the last group to maintain the growth rate of this group the same as that of the group fed the 4% casein diet. The test diets were fed for 40 to 50 days, as in experiment 1.

In experiment 3, the rats fed the 25% casein diet were pair-fed with the rats fed the 4% casein diet, and after feeding the test diets for 40 to 50 days, morphological changes in mitochondria were examined. Simultaneously, in this experiment, the effect of the addition of vitamins and phosphate to the 4% casein diet was examined. Rats were divided into 3 groups. One group was fed the 25% casein diet supplemented with only one-third the quantity of vitamin mixture as reported by Harper (19) and used in our previous experiments, and they were pair-fed with the group fed the 4% casein, low vitamin diet. Another group was fed the 4% casein diet supplemented with the amount of vitamins as reported by Harper (19) and supplemented further with 100 mg of vitamin E/kg of diet. In addition, in this experiment, two 4% casein diets were supplemented with 1.74 g of phosphorus/kg of diet so that the phosphorus uptake was the same as that for the 25% casein diet.

RESULTS

Body weight, liver weight and food intake. In table 2, the final body weight, liver weight and food intake are shown. Food intake was measured only in experiment 3. The weight of the rats fed the 4% casein diet changed only slightly. In experiment 2, the amount of the 25% casein diet fed to the restricted group was adjusted to prevent gain in body weight.

Oxidative activity using succinate as substrate. In table 3, results of oxygen consumption measurement in experiment 1 are shown. Also, in this table, oxygen consumption per milligram of protein at stages 3 and 4 and the respiratory control index are shown. The data were analyzed and the statistical significance of the difference between the groups fed the low and high casein diets was tested. To examine the effects of aging of mitochondria, oxygen consumption was again measured after putting the mitochondrial suspension in ice for 90 minutes. Measurement of oxygen consumption immediately after preparing mitochondrial suspension is termed period 1 and observation of the results obtained after aging is termed period 2.

TABLE 2

Final body weight, liver weight and food intake of rats fed 4% and 25% casein diets

Diet group	Treatment	No. rats/ group	Final body wt	Final liver wt	40-day food intake
			g	*g*	*g*
		Experiment 1			
4% casein [1]	ad libitum	3	167 ± 24 [2]	5.2 ± 0.7	
25% casein [1]	ad libitum	3	298 ± 23	9.9 ± 2.1	
4% casein [3]	ad libitum	3	163 ± 17	4.7 ± 0.4	
25% casein [3]	ad libitum	3	299 ± 19	10.2 ± 1.0	
		Experiment 2			
25% casein	ad libitum	5	260 ± 20	9.5 ± 1.2	
4% casein	ad libitum	5	143 ± 17	4.2 ± 0.6	
25% casein	restricted	5	141 ± 6	3.9 ± 0.4	
		Experiment 3			
4% casein	pair-fed	5	160 ± 14	5.0 ± 0.8	497 ± 33
25% casein	pair-fed	5	239 ± 19	7.4 ± 0.9	497 ± 33
4% casein + vitamins [4]	ad libitum	5	169 ± 9	4.9 ± 0.4	488 ± 35

[1] Bovine serum albumin was added to the preparation medium in an amount to make the final concentration in the medium 0.05%.
[2] Mean ± SD.
[3] Bovine serum albumin not added to preparation medium.
[4] Adequate vitamin supplement (19).

TABLE 3

Oxygen consumption and respiratory control index (exp. 1)

Diet group		Q_{O_2}[1]		Probability of difference of V_3 by *t* test between groups fed 4% and 25% casein diet	Respiratory control index (V_3/V_4)	Probability of difference of V_3/V_4 by *t* test between groups fed 4% and 25% casein diet
		V_3[2]	V_4[3]			
		With bovine serum albumin added				
Period 1	4% casein	219 ± 4 [4]	38 ± 1	$P < 0.01$	5.77 ± 0.14	$P < 0.01$
	25% casein	189 ± 16	37 ± 2		5.09 ± 0.17	
Period 2	4% casein	214 ± 5	38 ± 1	$P < 0.01$	5.68 ± 0.18	$P < 0.01$
	25% casein	170 ± 20	34 ± 3		4.85 ± 0.27	
		Without added bovine serum albumin				
Period 1	4% casein	229 ± 20	40 ± 8	$P < 0.05$	5.82 ± 0.66	$P < 0.1$
	25% casein	178 ± 23	40 ± 2		4.50 ± 0.53	
Period 2	4% casein	209 ± 18	40 ± 3	$P < 0.1$	5.28 ± 0.51	$P < 0.3$
	25% casein	178 ± 8	36 ± 1		4.87 ± 0.24	

[1] Value was calculated as microliters of O_2 absorbed per milligram of protein in one hour.
[2] V_3 represents value of O_2 consumption in the presence of ADP and succinate.
[3] V_4 represents value of O_2 consumption after the added ADP was used up, that is, the value of O_2 consumption in the presence of succinate.
[4] Mean ± SD.

Bovine serum albumin is known as an anti-swelling agent because of its nature of reacting with free fatty acids. In this experiment, the effect of the addition of bovine serum albumin in the preparation medium was examined.

Oxygen consumption per milligram of protein at stage 3 was greater in rats fed a low casein diet as compared with those fed a high casein diet (table 3). Although the difference was not great, it was statistically significant when bovine serum

albumin was added to the preparation medium, in both periods 1 and 2. When bovine serum albumin was not added, oxygen consumption was significant only in period 1. On the contrary, oxygen consumption at stage 4 was the same in groups fed the low casein and high casein diet in all cases. The respiratory control index was greater in the group fed the low casein diet, and the difference was significant in both periods 1 and 2 when bovine serum albumin was added, but not significant in either period without it. Effects of aging were noted slightly in the latter case.

Results of oxygen consumption measurement in experiment 2 are shown in table 4. In rats fed a 25% casein diet caloric restriction had no influence on mitochondrial oxidative activity. That is, oxygen consumption and the respiratory control index were the same for the 2 groups fed the high casein diet. These values were statistically significantly different from those for the rats fed the 4% casein diet.

Electron photomicrographs of isolated mitochondria. In figures 2 and 3, electron photomicrographs of isolated mitochondria from rats fed the 4% casein and 25% casein diets ad libitum are shown. These two electron photomicrographs were taken of the mitochondrial sample obtained after washing 6 times with the preparation medium. Swelling of the mitochondria isolated from rats fed the low casein diet was easily induced and the shape of the mitochondria was irregular as compared with the controls.

In figures 4 and 5 are shown, respectively, electron photomicrographs of mitochondria isolated from a rat fed the 4% casein diet and those from a rat pair-fed the 25% casein diet with the first rat. Phosphorus uptake was the same for both dietary treatments. Figure 5 shows that caloric restriction had no influence on the isolated mitochondria. Namely, the morphological change seen in mitochondria isolated from rats fed the 4% casein diet was due neither to lowered food intake nor to low phosphorus uptake. An electron photomicrograph of mitochondria isolated from a rat fed the 4% casein diet with the adequate vitamin supplementation (19) (fig. 6) shows that vitamin supplementation did not prevent the mitochondrial morphological change. The electron photomicrographs in figures 4, 5 and 6 were taken of the mitochondrial sample obtained after washing only once with the preparation medium.

DISCUSSION

In these experiments, the electron photomicrographs show clearly that swelling of liver mitochondria was easily induced by feeding rats a low casein diet.

Previous reports (12–18) indicate that under conditions of malnutrition, mitochondria were found to swell, and their oxidative and phosphorylative activity changed in almost all cases. In the case of diabetes, a decrease in oxygen consumption and P:O ratio has been reported (18). With essential fatty acid deficiency, an increase in oxygen consumption (14) and a decrease in P:O ratio (12–14) and

TABLE 4

Oxygen consumption and respiratory control index (exp. 2)

		Q_{O_2}[1]		Probability of difference of V_3 by *t* test	Respiratory control index (V_3/V_4)	Probability of difference of V_3/V_4 by *t* test
		V_3[2]	V_4[3]			
25% casein	ad libitum	128 ± 8 [4]	25 ± 2	$P < 0.01$ [5]	5.12 ± 0.69	$P < 0.01$ [5]
4% casein	ad libitum	174 ± 11	30 ± 3		6.32 ± 0.52	
25% casein	restricted	121 ± 22	24 ± 3	$P < 0.01$ [6]	5.00 ± 0.68	$P < 0.01$ [6]

[1] Value was calculated as microliters of O_2 absorbed per milligram of protein in one hour.
[2] V_3 represents value of O_2 consumption in the presence of ADP and succinate.
[3] V_4 represents value of O_2 consumption after the added ADP was used up, that is, the value of O_2 consumption in the presence of succinate.
[4] Mean ± SD.
[5] Probability of difference between 25% casein, ad libitum, and 4% casein, ad libitum, groups.
[6] Probability of difference between 4% casein, ad libitum, and 25% casein, restricted, groups.

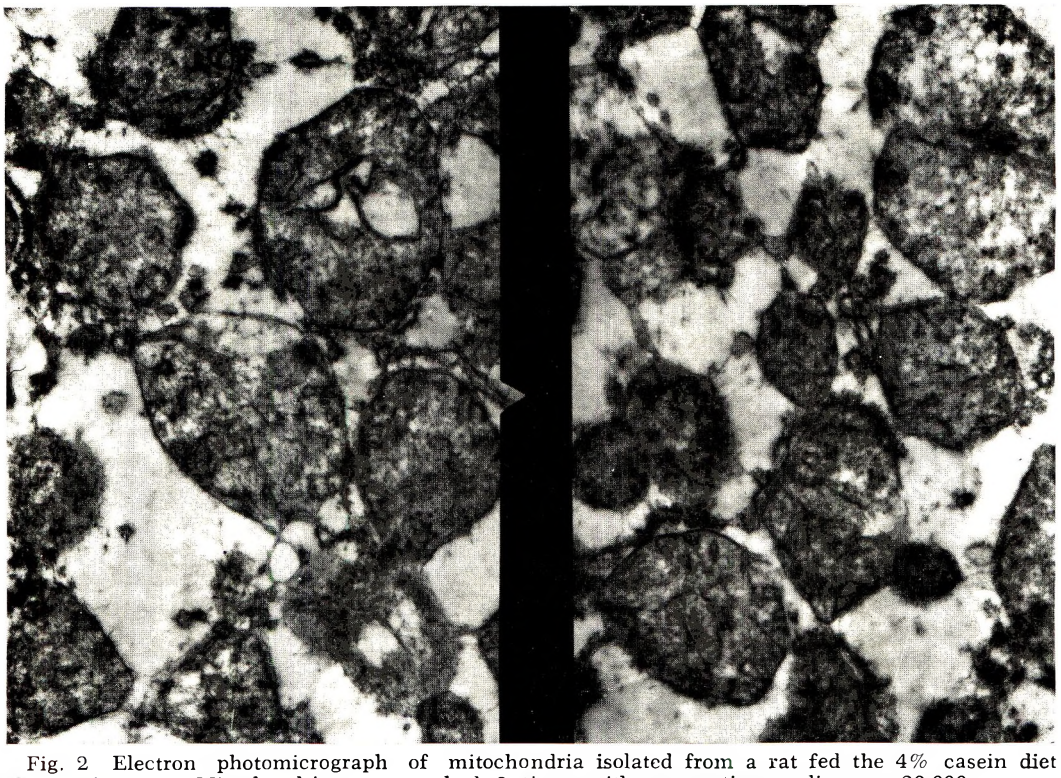

Fig. 2 Electron photomicrograph of mitochondria isolated from a rat fed the 4% casein diet in experiment 1. Mitochondria were washed 6 times with preparation medium. × 20,000.

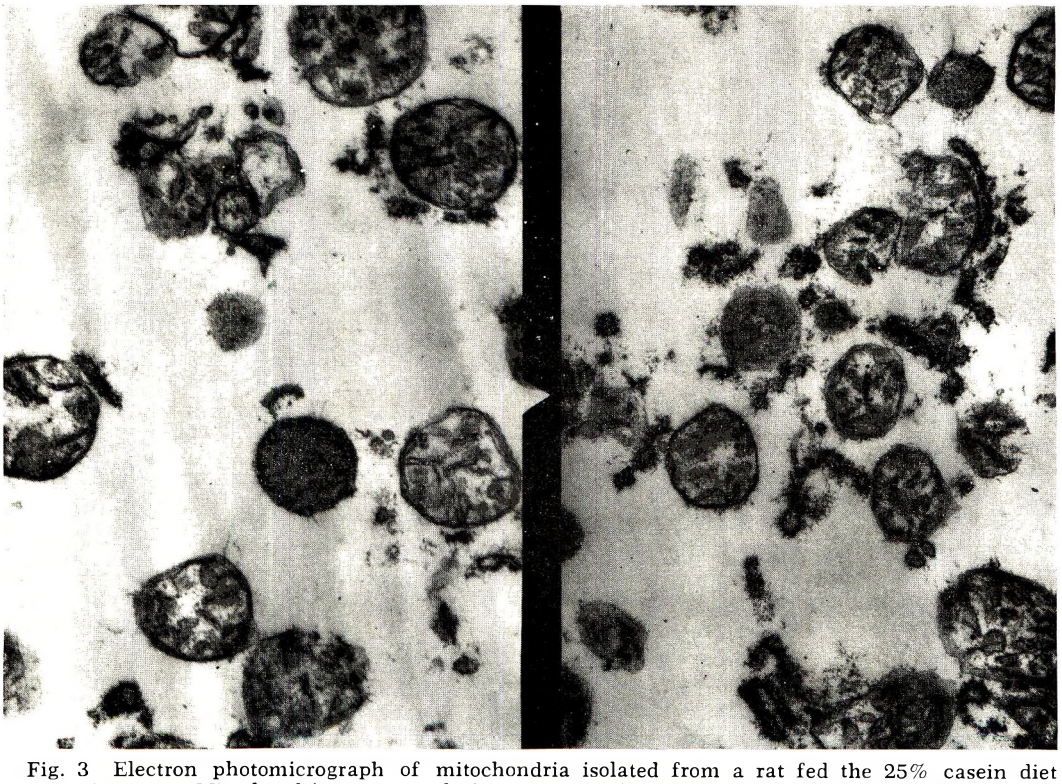

Fig. 3 Electron photomicrograph of mitochondria isolated from a rat fed the 25% casein diet in experiment 1. Mitochondria were washed 6 times with preparation medium. × 20,000.

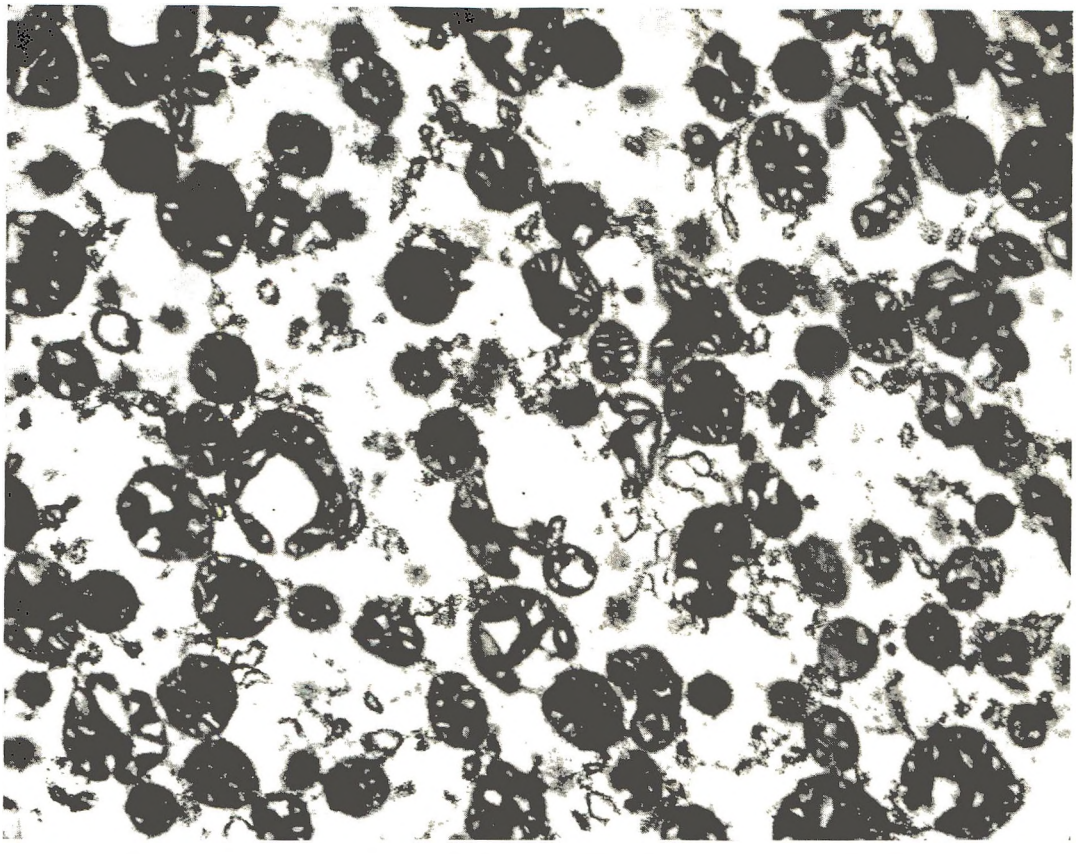

Fig. 4 Electron photomicrograph of mitochondria isolated from a rat fed the 4% casein diet in experiment 3. Mitochondria were washed only once with preparation medium, as when oxygen consumption was measured. × 15,000.

respiratory control index (17) have been reported.

In these experiments, measurement of oxygen consumption was made by the Warburg manometric method, except in the last case. But in order to measure the activity of the mitochondria retaining normal structure, the Warburg manometric method did not appear to be suitable, since the incubation is carried out while shaking, which may destroy the particulate structure. Moreover, to reach temperature equilibrium, previous shaking for a few minutes is necessary, and this might cause particle destruction during that time. If the mitochondria had a high phosphorylative activity in the early stage of preparation but were easily destroyed, then it would be impossible to measure the true values manometrically.

In the experiments reported here, the oxygen electrode was used instead of the Warburg manometric method. It was found that although liver mitochondria swelled in rats fed a low casein diet, their oxygen consumption and respiratory control index were higher than those in rats fed a high casein diet.

The results of experiment 3, show that vitamin supplementation had almost no effect on the mitochondrial change observed in rats fed a 4% casein diet, and vitamin E had no influence. Hence the mitochondrial change observed in rats fed the low casein diet was not due to low vitamin intake.

The food intake of rats fed a 4% casein diet was not low, averaging about 12 g /day (table 2). And the electron photomicrograph (fig. 5) shows that mitochon-

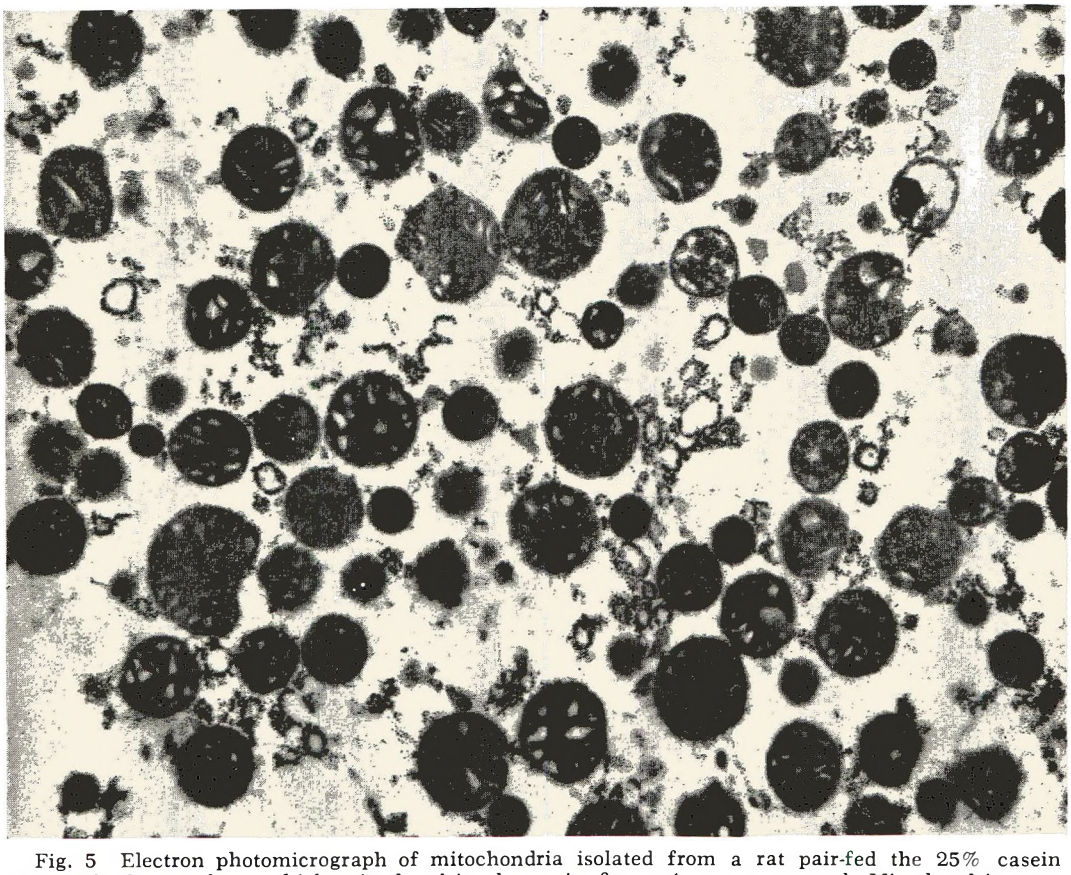

Fig. 5 Electron photomicrograph of mitochondria isolated from a rat pair-fed the 25% casein diet with the rat from which mitochondria shown in figure 4 were separated. Mitochondria were washed once with preparation medium. × 15,000.

dria of pair-fed control rats were not different from those of the normal rats. Nor was the oxidative activity for succinate in rats fed the severely restricted 25% casein diet different from that in the ad libitum group (table 4). It can be concluded, therefore, that the mitochondrial change observed in rats fed the 4% casein diet was not due to lowered food intake.

Casein was used as the dietary protein source in order that the relationship between any mitochondrial change observed and a deficiency of sulfur-containing amino acids or protein can be investigated in a future study.

It has been reported (26, 27) that an increase in free fatty acids causes swelling of mitochondria. The effect of an increase in free fatty acids should be investigated further since bovine serum albumin affects the respiratory control index slightly with aging of the mitochondria.

The reason for the observed swelling of liver mitochondria when rats were fed a low casein diet is not understood. But despite the swelling of the mitochondria, the respiratory control index was rather high. This may have resulted from using the oxygen electrode instead of a Warburg manometer. These studies indicate that under such an abnormal dietary condition, some biochemical changes do occur in mitochondria.

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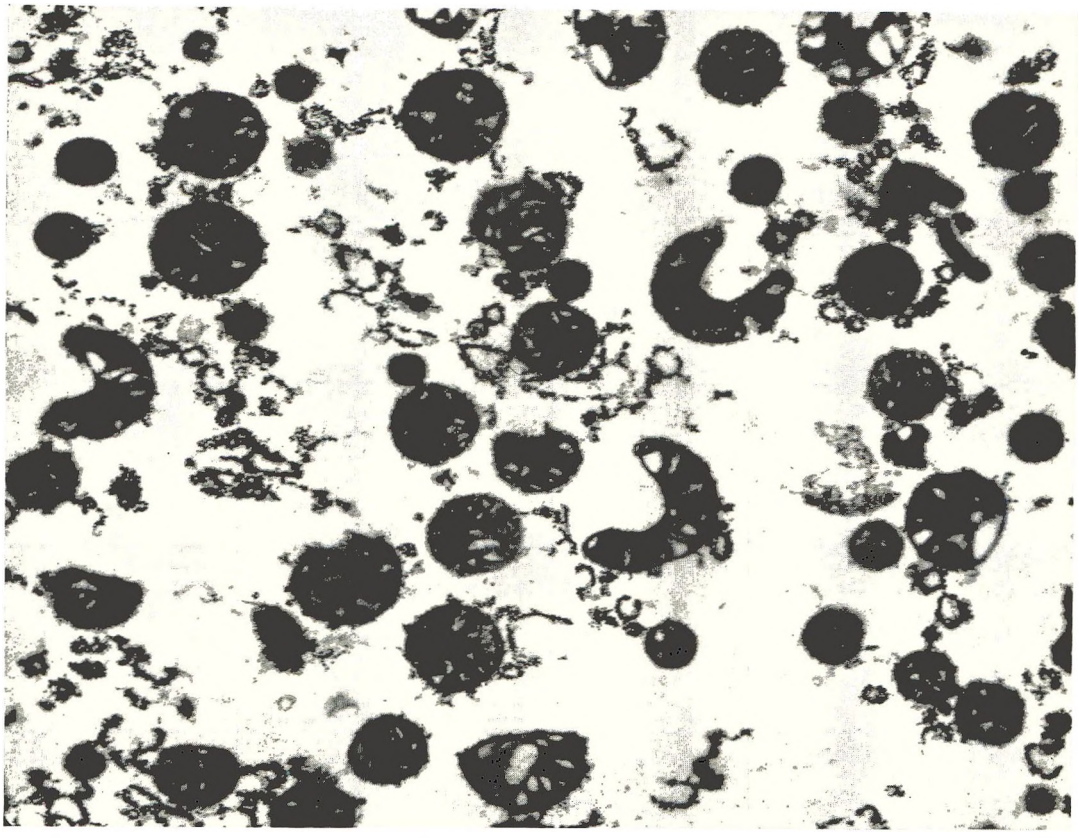

Fig. 6 Electron photomicrograph of mitochondria isolated from a rat fed the 4% casein diet with adequate vitamin supplement (19). Mitochondria were washed once with preparation medium. × 15,000.

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Effect of Dietary Calcium, Buffering Capacity, Lactose and EDTA on pH of and Calcium Absorption from Gastrointestinal Segments in the Growing Rat [1]

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ABSTRACT The effects of dietary calcium, buffering capacity (BC), lactose and ethylenediaminetetraacetic acid (EDTA) on the pH pattern of and calcium absorption from 6 segments of the gastrointestinal tract were studied. One hundred and forty-four rats were used for a replicated, factorially arranged experiment, which involved calcium (0.19, 0.48 and 0.78%), BC 1) 0.46 ml, 2) 0.54 ml and 3) 0.76 ml N HCl required to reduce the pH of 5.0 g of the diet by one pH unit), lactose (0 and 12%), and EDTA (0 and 0.2%). Chromic oxide served as a reference substance for calcium absorption. The increased calcium absorption with decreased calcium intake was a result of increased hydrogen ion concentration in the intestinal secretion; decreased endogenous secretion of calcium in the duodenum which increased dietary calcium absorption in the lower gastrointestinal tract; and increased calcium absorption all along the intestinal tract posterior to the duodenum. BC controlled the pH of gastrointestinal contents and lactose produced a more acid pH in the ileum. The percentage calcium absorption increased with the more intestinal acid pH, and decreased with dietary increases in calcium, BC or EDTA. Calcium and BC of the diet interacted and influenced calcium secretion in the duodenum and calcium absorption in the jejunum and ileum.

As a group, divalent ions are absorbed less readily from the intestinal lumen than are most monovalent ions. Studies in vivo and in vitro have shown that percentage as well as total calcium absorption was dependent on dietary calcium (1–4). The relationship between solubility and availability of calcium for absorption has been disputed for years (5). While increased acidity in the stomach was reported to increase calcium absorption (6–8), the probability exists that the contents of the intestine are well-buffered, that such changes in pH are compensated for rapidly (4). Lactose was reported to result in decreased ileal pH and increased calcium absorption in the intestine (9–13). The chelate, calcium ethylendiaminetetraacetate (EDTA), was found to be unavailable to the rat from the intestine (14),[3] and dietary EDTA did not affect calcium absorption after a 12-week adaptation period (15).

The present study reports the main effects and the interrelationships of 4 factors, calcium, buffering capacity (BC), lactose and EDTA, on pH of and calcium absorption from 6 segments of the gastrointestinal tract.

PROCEDURE

A randomized block design using 4 replicates with a $3 \times 3 \times 2 \times 2$ factorial arrangement of treatments was used so that all possible combinations of 3 levels of calcium, 3 BC's, and 2 levels each of lactose and EDTA were fed to growing rats. Levels were 0.19, 0.48 and 0.78% calcium, zero and 12% lactose, zero and 0.2% EDTA, with 0.4% phosphorus for each of the 36 diets. The BC's were 0.46, 0.54 and 0.76 and were obtained by varying the dihydrogen to monohydrogen phosphate ratios from 1:0 (BC 1) to 1:1 (BC 2) to 1:2 (BC 3), respectively. Dietary BC was defined as milliliters of N HCl required to reduce the pH of 5.0 g of the diet suspended in 45 ml of distilled deionized water by one pH unit.

Hooded Norway male rats ranging in weight from 70 to 90 g were adjusted to a mixture of laboratory animal ration and

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the basal diet for 1 week before feeding the appropriate experimental diets, compositions of which are shown in table 1. The experimental diet and distilled deionized water were supplied ad libitum for the 6-week experimental period and chromic oxide was added to each diet to serve as a reference substance for the determination of calcium absorption (8, 16). Rats were housed individually in stainless steel cages in a room maintained at 25 ± 1° and 57 ± 3% humidity.

One hour after the diets were removed from the cages, the rats were killed and the gastrointestinal tracts were removed and divided according to Marcus and Lengemann (17) into 6 segments: the stomach; four equal parts of the small intestine, representing duodenum, jejunum, jejunum-ileum and ileum; and the cecum. The contents of each segment were transferred quantitatively into 50-ml Polyethylene containers, made to a constant volume, and the pH was determined by means of a combination electrode. The contents were then frozen and kept in this state until analyzed for calcium and chromic oxide. The dried gastrointestinal tract contents were ashed with nitric acid, as suggested by Czarnocki et al. (18), oxidized with perchloric acid and sodium molybdate mixture, and transferred to a volumetric flask. The optical density for chromic oxide was recorded at 440 mμ (19), and calcium was determined according to Steckel and Flannery.[4] The ratio of calcium to chromic oxide in the contents of the 6 gastrointestinal segments was used to calculate the relative apparent absorption or secretion for each segment (16). The following equation was used: % absorption (+) or secretion (−) in segment 2 =

$$\left(100 - \frac{\%\text{ chromic oxide in segment 1}}{\%\text{ chromic oxide in segment 2}} \times \frac{\%\text{ calcium in segment 2}}{\%\text{ calcium in segment 1}} \times 100\right).$$

To calculate the absorption or secretion in the different segments of the gastrointestinal tract as the digesta moved posteriorly, the digesta in a given segment was considered as the diet (segment 1) for the next posterior segment (segment 2). In this way the diet represented segment 1 and the stomach, segment 2. Continuing posteriorly, the segments following the stomach were the duodenum, jejunum, jejunum-ileum, ileum and cecum.

TABLE 1

Composition of basal diet

	%
Sucrose	30.0
Starch	30.0
Casein	20.0
DL-Methionine	0.2
Corn oil [1]	5.0
Vitamin mixture [2,4]	2.0
Micromineral mixture [3,4]	0.03
Macromineral mixture (BC 1, 2 or 3) [5]	12.77

[1] Santoquin (Monsanto Company, St. Louis) added to corn oil to make 0.01% in diet.

[2] The vitamins mixed with dextrose supplied the following per 100 g of diet: vitamin A, 1800 IU; vitamin D, 200 IU; and (in milligrams) α-tocopherol, 10.0; ascorbic acid, 90.0; inositol, 10.0; choline chloride, 150.0; riboflavin, 2.0; menadione, 4.5; *p*-aminobenzoic acid, 10.0; niacin, 9.0; pyridoxine·HCl, 2.0; thiamine, 2.0; Ca pantothenate, 6.0; biotin, 0.04; folic acid, 0.18; and vitamin B_{12}, 0.003.

[3] Micromineral mixture supplied the following per 100 g of diet: (in milligrams) iron as Fe_2O_3, 8.58; manganese as $MnCO_3$, 4.0; zinc as $ZnCO_3$, 5.0; copper as $CuCO_3$, 1.17; cobalt as $CoCO_3$, 0.04; molybdenum as $Na_2MoO_4 \cdot 2H_2O$, 0.06; iodine as KI, 0.02; selenium as H_2SeO_3, 0.01; fluoride as NaF, 0.001.

[4] NRC (29) requirements were met.

[5] Evans and Ali (30).

RESULTS AND DISCUSSION

The effect of graded levels of dietary calcium on gastrointestinal absorption of calcium is one of the best examples for demonstrating the ability of the animal body to adjust its metabolic activities to satisfy body needs. Differences in dietary calcium resulted in differences in pH in the stomach (quadratic, $P < 0.05$) and the intestinal tract (linear, $P < 0.01$). It was reported that a decreased pH increased the concentration of ultra-filtrable, soluble, and non-bound form of calcium (7, 20). The reduced gastrointestinal pH with 0.19% dietary calcium intake is a possible mechanism of adaptation which increased the solubility of calcium and hence the absorption of calcium (table 2).

The stomachs of the pig and the rat and the abomasum of the calf participate in calcium absorption (16, 21, 22). Decreased dietary calcium caused changes (linear and quadratic, $P < 0.01$) in calcium absorption in the stomach. With the diet containing 0.19% calcium, absorption in the stomach was detected, and with the

[4] Steckel, J. E., and R. L. Flannery 1965 Automatic determination of phosphorus, potassium, calcium and magnesium in wet digestion solutions of plant tissue. Technicon Symposium, Technicon Instrument Company Publications, Chauncey, New York.

TABLE 2

The pH of gastrointestinal tract segment contents for main effects of dietary variables

Segment	Calcium, %			Buffering capacity [1]			EDTA, %		Lactose, %		SE
	0.19	0.48	0.78	0.46	0.54	0.76	0	0.2	0	12.0	
Stomach	4.2	4.1	4.3	3.3 [2]	4.3	4.9	4.2	4.2	4.2	4.2	0.06
Duodenum	5.8 [2]	6.0	6.2	6.2 [2]	6.0	5.8	6.0	6.0	6.0	6.0	0.02
Jejunum	6.0 [2]	6.3	6.4	6.4 [2]	6.3	6.2	6.2	6.2	6.3	6.2	0.02
Jejunum-ileum	6.3 [2]	6.6	6.8	6.4 [2]	6.6	6.7	6.5	6.6	6.6	6.5	0.03
Ileum	6.7 [2]	7.0	7.2	6.6 [2]	7.0	7.3	6.9	7.0	7.2 [3]	6.7	0.04
Cecum	6.4 [2]	6.8	7.0	6.6 [2]	6.7	6.8	6.7	6.8	7.0 [3]	6.4	0.04

[1] Milliliters of N HCl required to reduce pH of 5 g of mixed diet by 1 pH unit.
[2] Significant linear main effect ($P < 0.01$).
[3] Significant main effect ($P < 0.01$).

diet containing 0.78% calcium, secretion was detected (fig. 1 and table 3). The participation of the stomach in calcium absorption depended on the level of dietary calcium. The process appeared to be active, since if it took place by diffusion alone calcium absorption would be expected to increase with increased calcium concentration in the lumen of the stomach.

Under our experimental conditions no calcium absorption was detected in the duodenum. Duodenal calcium secretion increased 80% with increases of dietary calcium from 0.19 to 0.78% (linear and quadratic, $P < 0.01$). The main function of the duodenum was the secretion of calcium, and this secretion was related to the level of calcium intake (fig. 1 and table 3). That calcium absorption was not detected in the duodenum could be due to the dilution factor of the pancreatic enzymes and bile secretion on the digesta and to the fast rate at which the digesta traverse the duodenum (17).

The presence of an endogenous factor that acts to control calcium absorption in the intestine to meet the body needs was suggested (24). It was reported that there was a carrier system involved in calcium transport across the intestinal wall (25); the capacity of this system to transport calcium, however, was limited, and a protein that was isolated and identified from the intestinal mucosa of non-rachitic chicks was found to be absent from the intestinal mucosa of rachitic chicks (26). This protein and the calcium-carrier system could be one and the same. If this carrier system theory should prove to be valid, then an ample secretion of endogenous calcium in the upper part of the intestinal tract would be the best device to control absorption of excess dietary calcium in the lower part of the intestine.

With 0.19% dietary calcium, calcium absorption was detected all along the gastrointestinal tract posterior to the duodenum (linear, $P < 0.01$); and with 0.78% dietary calcium, this absorption was concentrated mainly in the ileum (linear, $P < 0.01$ and quadratic, $P < 0.05$, fig. 1 and table 3). These observations were in agreement with those arrived at by the inverted gut sac technique (27). It was reported that endogenous calcium was more available for absorption in the ileum than dietary calcium (23), which would explain the higher calcium absorption in the ileum with 0.78% than with 0.19% dietary calcium because of the larger duodenal calcium secretion with 0.78% calcium.

The diets (BC 3) most resistant to acidification in the stomach (linear, $P < 0.01$) were the last to be neutralized in the duodenum and jejunum (linear $P < 0.01$, table 2). This appears to indicate that the BC 3 diet stayed longer and consumed more acid in the stomach and this delayed neutralization in the duodenum. To balance the amount of HCl secreted with BC 3 diets in the stomach more base had to be secreted in the posterior segments of the intestinal tract. The diets which were most acid in the stomach (BC 1) maintained the lowest pH posterior to the jejunum (linear, $P < 0.01$, table 2).

Calcium absorption with BC 1 diets started in the stomach where an acidic condition prevailed while calcium secretion was taking place with BC 3 diets (linear, $P < 0.01$, fig. 1 and table 3). Considering the ileum as the main site of calcium absorption (17, 21), and considering

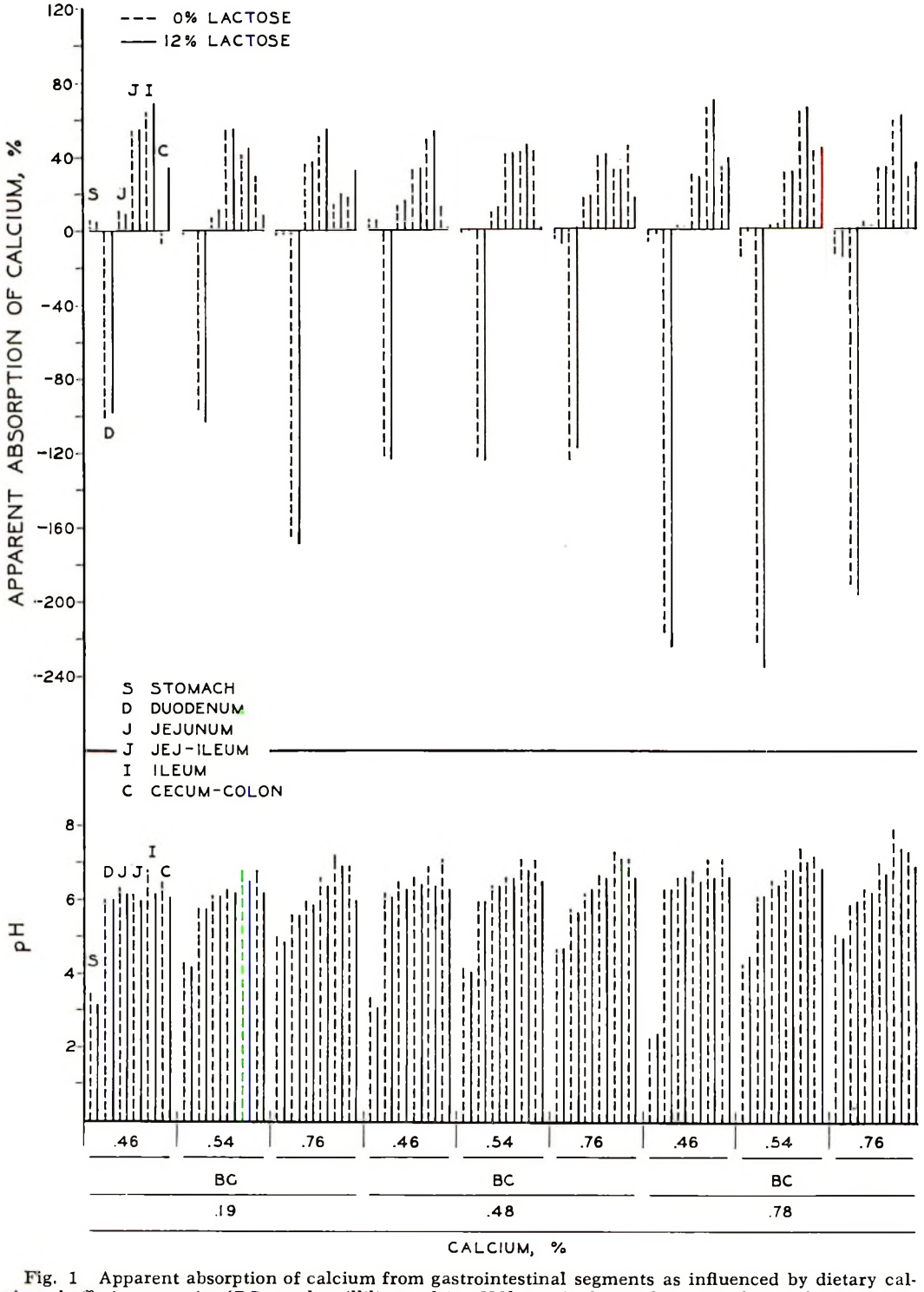

Fig. 1 Apparent absorption of calcium from gastrointestinal segments as influenced by dietary calcium, buffering capacity (BC equals milliliters of 1 N HCl required to reduce pH of 5 g of mixed diet by 1 pH unit), and lactose.

TABLE 3

Relative percentage absorption (+) and secretion (−) of calcium by segments of the gastrointestinal tract for main effects of dietary variables

Segment	Calcium, %			Buffering capacity [1]			EDTA, %		Lactose, %		SE
	0.19	0.48	0.78	0.46	0.54	0.76	0	0.2	0	12.0	
Stomach	1.0 [2]	−0.6	−8.0	2.1 [2]	−2.1	−7.9	−2.7	−2.5	−3.3	−1.9	0.8
Duodenum [3]	−122.5	−123.0	−215.0	−148.0	−149.1	−163.3	−153.2	−153.9	−151.8	−153.3	4.5
Jejunum [3]	18.8	14.4	1.8	8.7	7.4	18.9	11.5	11.8	11.2	12.2	1.0
Jejunum-ileum	53.9 [2]	38.1	30.8	38.8	42.2	41.7	40.8	40.9	40.5	41.4	1.0
Ileum [3]	42.1	42.4	63.9	62.0	50.1	36.1	49.8	49.1	48.3 [4]	50.6	1.5
Cecum	19.4	19.6	36.8	18.9	27.6	29.3	24.1	26.3	27.1	23.4	2.9

[1] Milliliters of N HCl required to reduce pH of 5 g of mixed diet by 1 pH unit.
[2] Significant linear main effect ($P < 0.01$).
[3] Significant ($P < 0.01$) calcium and buffering capacity (BC) interaction for calcium absorption and secretion (−).

A. Duodenum

Ca, %	BC 1	BC 2	BC 3
0.19	−100	−100	−167
0.48	−123	−124	−122
0.78	−221	−230	−195

B. Jejunum

	BC 1	BC 2	BC 3
	10	10	37
	15	10	18
	1	2	3

C. Ileum

BC 1	BC 2	BC 3
67	43	17
52	44	32
67	64	60

[4] Significant main effect ($P < 0.05$).

that there was no significant difference in endogenous calcium secretion in the duodenum among the three dietary BC's (fig. 1), it was concluded that the increase in calcium absorption in the ileum with BC 1 (linear, $P < 0.01$) was due to the prevailing acidic pH. The increased availability of calcium for absorption in the stomach and ileum with decreased pH is considered an indication of a relationship between increased solubility and availability of calcium for absorption in the gastrointestinal tract.

The segments of the intestine where lactose showed an effect on acidity and calcium absorption were the ileum and the cecum ($P < 0.01$, fig. 1 and table 2). Feeding lactose decreased the pH of the ileum and the cecum and increased calcium absorption in the ileum ($P < 0.05$). Individual variation in calcium absorption in the cecum was so large that it obscured any possible difference due to lactose feeding. The anatomical relationship of the cecum and the sluggishness of cecal movement (28) would likely allow separation of heavy molecules such as chromic oxide in the cecum which upset the calcium to chromic oxide ratio in the cecal contents. These facts should be kept in mind and expected whenever a heavy molecule such as chromic oxide, yttrium, or titanium dioxide is used as an absorption indicator.

Previously (30), it was determined that a diet with 0.2% EDTA fed for 6 weeks reduced total body and femur calcium. With 0.2% EDTA in the diet there was no reduction in calcium absorption (table 3) at the end of the 6-week period which would be expected if the calcium was trapped as an unavailable chelate. Thus, it was concluded that the effect of EDTA on calcium absorption is dependent on the time allowed for adaptation to a diet containing EDTA and on the calcium and the EDTA content of a diet.

The direct effect of dietary calcium, BC, and lactose on the pH of different segments of the gastrointestinal tract and on calcium absorption caused several 2-factor interactions. Dietary calcium and BC interacted at the duodenum, jejunum and ileum (table 3). The most efficient dietary calcium absorption was noticed with the combination of 0.19% calcium

and BC 1 where duodenal secretion of calcium was the least with the maximal level of calcium absorption in the ileum. The interaction between dietary BC and lactose ($P < 0.05$) on calcium absorption in the ileum suggested a possible closer relationship between the enhancing effect of lactose on calcium absorption and pH of the ileum.

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Complex Formation of Allylamine with Pyridoxal Phosphate and Inhibition of GO-T and GP-T of Human Serum and Rat Liver by Allylamine

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ABSTRACT From absorption spectra, it was confirmed that allylamine forms a complex with pyridoxal phosphate in both acid and basic solution. Allylamine inhibited the activity of human serum glutamic-oxaloacetic transaminase (GO-T) and glutamic-pyruvic transaminase (GP-T) in vitro at the same concentration as isonicotinic acid hydrazide acting as anti-vitamin B_6. The liver GO-T and GP-T activities were significantly lower in allylamine-treated rats than in control rats. From these results, it is suggested that allylamine has an inhibitory effect on the vitamin B_6-dependent enzymes.

It has been reported by Reinhart and Greenberg (1) that pyridoxine deficiency in the rhesus monkey induces arteriosclerosis similar to that occurring naturally in man. Previously, the authors had reported the recovery of pyridoxine-deficient monkeys from arteriosclerosis following administration of vitamin B_6 (2). It has also been demonstrated that the administration of allylamine to dogs causes an injury of the arterial wall and the development of arteriosclerosis (3). Although the etiology of these cases of arteriosclerosis has not been clearly elucidated, it has been shown that arteriosclerosis can be produced without hyperlipemia.

From the viewpoint that both allylamine and pyridoxine have an apparent connection with the development of arteriosclerosis, the present study was made to clarify the relationship between allylamine and pyridoxal phosphate.

EXPERIMENTAL

Preparations for the complex formation of allylamine with pyridoxal phosphate were carried out as follows: 3 solutions were prepared at pH 5.2, 7.4 and 8.0, respectively: solution 1, pyridoxal phosphate, 10 μg/ml in 0.067 M phosphate buffer; solution 2, pyridoxal phosphate, 10 μg/ml and allyamine, 1 mg/ml in 0.067 M phosphate buffer; and solution 3, allylamine 1 mg/ml in 0.067 M phosphate buffer. Analyses of the solutions were carried out on a Beckman spectrophotometer.

The serum of a patient suffering from infectious hepatitis, which was shown to have a high activity of glutamic-oxaloacetic transaminase (GO-T) and glutamic-pyruvic transaminase (GP-T), was diluted to the proper concentration for measurement. Allylamine was added to the reaction mixture at a final concentration of 1×10^{-5} M. The activity of the GO-T and GP-T was measured by the method of Reitman and Frankel (4). Simultaneously the effect of isoniazid (isonicotinic acid hydrazide, INH), which has been reported to inhibit the GO-T and GP-T by complexing with pyridoxal phosphate (5), was demonstrated to be comparable to that of allylamine.

To determine the effect of allylamine on the liver GO-T and GP-T activities in rats, 10 male rats of the Wistar strain, weighing 130 to 150 g, were divided into 2 groups of five each. To one group allylamine dissolved in 0.4 ml of saline was injected into the caudal vein at a level of 60 mg/kg body weight. Another group was injected with 0.4 ml of saline as control. Five minutes after injection, the animals were killed; the livers were excised and 1 g of liver was homogenized in 0.25 M sucrose solution, the final volume of the homoge-

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nate being brought to 1000 ml (0.1%/vol homogenate). The activities of the GO-T and GP-T were measured by the method of Reitman and Frankel (4) using 0.1 ml of homogenate. (The units of value by this method correspond to the Karmen unit. According to Karmen, one unit is determined to correspond to 0.001 of optical density decrease in one minute under the condition that the reaction occurred at room temperature, 23° to 26°.)

RESULTS

Complex formation of allylamine with pyridoxal phosphate. As shown in figures 1–3, the absorption spectrum of the mixture of pyridoxal phosphate and allylamine is different from that of pyridoxal phosphate or allylamine alone at pH 5.2, 7.4 and 8.0. These results indicate that allylamine forms a complex with pyridoxal phosphate in both the acid and the basic solution.

Inhibition of the serum GO-T and GP-T by allylamine. The activity of the serum GO-T was inhibited by allylamine at a concentration of 1×10^{-2} M, while that of the serum GP-T was inhibited at a concentration of 1×10^{-4} M (table 1). Allylamine inhibited the serum GP-T at a lower concentration than in the case of the GO-T. Similar results were obtained with INAH.

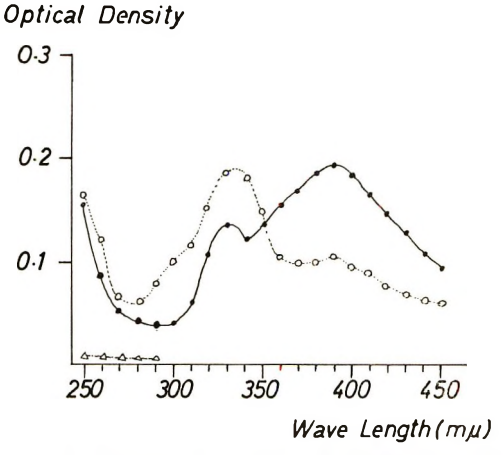

Fig. 1 Complex formation of allylamine with pyridoxal phosphate in vitro (pH 5.2). Key: ● —— ●, pyridoxal phosphate, 10 μg/ml and allylamine 1 mg/ml in 0.067 M phosphate buffer; ○ .... ○, pyridoxal phosphate 10 μg/ml in 0.067 M phosphate buffer; and △ – · – △, allylamine 1 mg/ml in 0.067 M phosphate buffer.

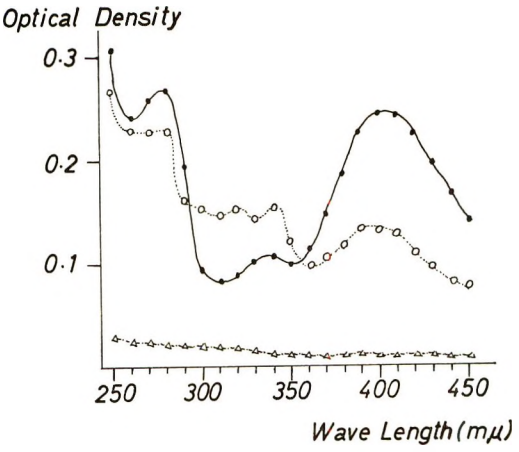

Fig. 2 Complex formation of allylamine with pyridoxal phosphate in vitro (pH 7.4); see legend of figure 1 for key.

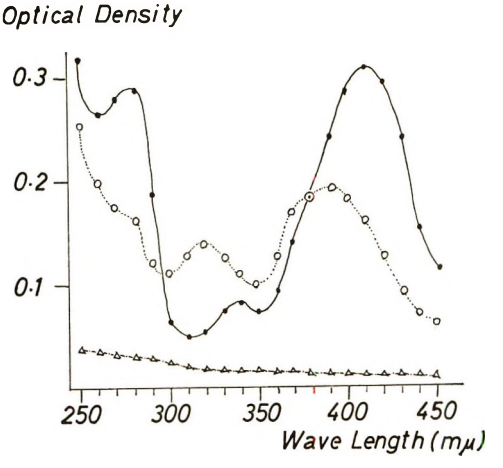

Fig. 3 Complex formation of allylamine with pyridoxal phosphate in vitro (pH 8.0); see legend of figure 1 for key.

Effect of allylamine on the liver GO-T and GP-T activities in rats. As shown in table 2, the liver GO-T and GP-T activities were lower in allylamine-treated rats than in the controls. According to Student's *t* test, the differences between 2 groups were statistically significant ($P < 0.01$).

DISCUSSION

It is generally recognized that INH forms the hydrazone complex with pyridoxal phosphate to inactivate the co-

TABLE 1

Inhibition of the serum GO-T and GP-T by allylamine or INAH in vitro [1]

	Final conc. of inhibitor	GO-T [2]		GP-T [2]	
		Units	Inhibition	Units	Inhibition
	M	*units/ml*	%	*units/ml*	%
		Allylamine			
	1	17.5	82.5	0	100
	10^{-1}	35.0	65.0	25.0	81.1
	10^{-2}	77.5	22.5	32.5	75.5
	10^{-3}	94.0	6.0	105.0	20.8
	10^{-4}	95.0	5.0	115.0	13.2
		INAH			
	10^{-1}	10.0	90.0	0	100
	10^{-2}	72.5	27.5	47.5	64.2
	10^{-3}	97.5	2.5	102.5	22.6
Control	0	100.0	—	132.5	—

[1] Allylamine or INAH was added to the reaction mixture in vitro.
[2] See Experimental section for definition of units.

TABLE 2

Inhibition of the liver GO-T and GP-T in rats by allylamine [1,2]

	GO-T [3]	GP-T [3]
	$\times 10^3$ *units/g*	$\times 10^3$ *units/g*
Control	67.8 ± 6.1 [4]	22.8 ± 3.7
Allylamine	37.8 ± 10.3 *	11.4 ± 4.5 *

[1] The activities of the liver GO-T and GP-T were measured 5 minutes after injection of allylamine.
[2] Five animals/group.
[3] See Experimental section for definition of units.
[4] SD.
* Statistically significant ($P < 0.01$).

enzyme. Killam and Bain (6) showed that this substance induced seizures and decreased levels of γ-aminobutyric acid resulting from the decreased activity of the glutamic acid decarboxylase.

While the binding of pyridoxal phosphate with the glutamic acid decarboxylase is loose, that between pyridoxal phosphate and transaminase is tight (7). Therefore, there is less inhibition of the transaminase by INH. Sass and Murphy (8), however, demonstrated that 500 mg of INH daily lowered the GO-T in human beings whereas 300 mg did not. Hashimoto and Iwamoto (9) reported that INH blocked the liver GO-T activities of rats both in vitro and in vivo.

In the present experiments the authors demonstrated that allylamine formed a complex with pyridoxal phosphate both in the acid and basic solution and inhibited the serum GO-T and GP-T activity in vitro at the same concentration as INH did and also that the liver GO-T and GP-T activity were decreased in allylamine-treated rats.

From these results it is suggested that allylamine has an inhibitory effect on the vitamin B_6-dependent enzyme.

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Effect of Ration upon the Intestinal Distribution of Ca, Mg, Na, K and N in Calves [1]

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ABSTRACT The intestinal distribution of Ca, Mg, Na, K and N was compared in 3-month-old calves fed semipurified, concentrate and concentrate + hay rations. Unabsorbed markers (Cr_2O_3 or ^{144}Ce) were used to determine net secretion or absorption, or both, of Ca, Mg, Na, K and N along the gastrointestinal tract. All calves were fed constant amounts of their respective ration and an unabsorbed marker for at least 8 days before killing. Calves were killed 4 hours after feeding and their gastrointestinal tracts tied off, removed, divided into sections, and the contents weighed and sampled. A net secretion of Ca, Mg, Na, K and N occurred in the upper small intestine in all calves. Absorption of these constituents was greatest in passage through the small intestine. Few changes occurred in the intestinal concentrations of Ca, Mg and N in passage through the cecum and large intestine. Minimal values of Na and K secretion and absorption were calculated for the lower gut. It was estimated that an average of 133 g of Na were secreted into the upper small intestine daily. During passage through the lower gut, 87% of the net Na absorption occurred in the small intestine and 13% in the cecum and large intestine. The minimal value of K secretion into the upper small intestine was estimated to be 36 g daily. Absorption of K from the small intestine accounted for 90% of the net K absorption from the lower gut. Differences attributable to ration were observed in the ruminal concentrations of Mg and K in the calves fed the semipurified ration. Few concentration differences were evident in the small intestine that could be attributed to ration.

The concentrations of many minerals have been determined at various locations along the gastrointestinal tract of ruminants. The ultrafiltrable concentration of Ca and Mg have been measured in gut contents of slaughtered cows (1) and anesthetized sheep (2). van't Klooster (3) determined the concentration of Na, K, Ca and Mg in intestinal contents by a dialysis procedure using fistulated sheep.

The influx and efflux of minerals throughout the gastrointestinal tract does not permit the quantitative expression of absorption or secretion of a particular constituent. Therefore, a commonly used method for determining net absorption or secretion has been to compare intestinal constituent concentrations to the concentration of an unabsorbed marker. The sites of absorption of Ca and P (4), I (5) and Zn (6) have been determined in calves through the simultaneous administration of an unabsorbed marker with the feed and the respective isotope.

The small intestine transit time affects the efficiency of Mg absorption (7) and the vitamin D status of the animal affects the efficiency of Ca absorption (8). The factors defining the efficiency of Na and K absorption in the small intestine are less clear. Information on the effect of the electrolyte concentration in the feed on the subsequent concentration in the ingesta is limited. Smith (9) reported that differences in Na or K intake had no direct effect on their ileal emergence rates in calves fitted with re-entrant fistulas. Yang and Thomas (10) measured many organic and inorganic constituents in intestinal contents of calves fed rations that contained different levels of fiber. Their results indicated that the type of ration (high or low fiber) did not greatly affect the intestinal Ca or P concentrations. Mraz and Patrick (11) demonstrated that the type of diet fed to rats influenced the K excretory pattern. The present investigation was undertaken to determine the effect of extremely

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different rations upon the electrolyte distribution in intestinal contents of calves.

PROCEDURE

Eleven Holstein calves averaging 150 kg were divided into 3 groups. Group A (4 calves) was fed a semipurified ration similar to one used successfully for lambs (12) (table 1). Group B (4 calves) was fed a concentrate ration that consisted of 76.5% ground ear corn, 5.0% alfalfa meal, 12.0% cottonseed meal, 5.0% molasses and 0.5% each of bone meal, dicalcium phosphate and trace mineralized salt.[3] Group C (3 calves) was fed a ration that consisted of the above concentrate mixture and alfalfa hay. The phosphorus content was 0.38%, 0.35% and 0.31% for the rations consumed by group A, B, and C, respectively. Twice daily all the calves were fed constant amounts of their respective feed. The amount of feed offered at each feeding was determined initially by the amount they would consume within one hour during the adjustment period. Immediately before each feeding groups A and C received 25 μCi of ^{144}Ce as an unabsorbed marker in a gelatin capsule. Each calf in group B received 0.5 g of Cr_2O_3 per feeding as an unabsorbed marker. The Cr_2O_3 was mixed with 10 g of starch which was then mixed into the feed that was offered per feeding. It has been established that ^{144}Ce is comparable to Cr_2O_3 when used as an unabsorbed marker (13).

TABLE 1

Composition of semipurified ration [1]

	%
Casein	26.5
Glucose	31.8
Cornstarch	17.7
Hydrogenated vegetable oil	3.5
$KHCO_3$	4.4
$NaHCO_3$	7.3
Vitamin mixture [2]	4.4
Mineral mixture [3]	4.4

[1] Matrone et al. (12).

[2] Vitamin mixture (2.27 kg); thiamine·HCl, 400 mg; riboflavin, 850 mg; nicotinic acid, 1.13 g; Ca pantothenate, 1.42 g; pyridoxine·HCl, 570 mg; folic acid, 57 mg; *p*-aminobenzoic acid, 1.13 g; inositol, 11.35 g; biotin, 11.4 mg; choline chloride, 113.45 g; menadione, 115 mg; 0.1% vitamin B_{12} in mannitol, 4.66 g; α-tocopheryl acetate, 570 mg; glucose, 2132 g; 4000 IU of vitamin A and 400 IU of vitamin D administered/day/45 kg body wt via capsules.

[3] Mineral mixture (2.27 kg): $CaHPO_4$, 818 g; KCl, 273 g; NaCl, 239 g; $MgSO_4$, 204 g; $CuSO_4 \cdot 5H_2O$, 893 mg; $FeSO_4 \cdot 2H_2O$, 7648 mg; $MnSO_4 \cdot H_2O$, 1399 mg; ZnO, 2263 mg; $CoCO_3$, 9 mg; KI, 6 mg; and glucose, 722 g.

The calves were confined in metabolism stalls for a preliminary adjustment period (3 weeks for group A and 2 weeks for groups B and C) and for administration of the unabsorbed markers. On the morning the calves were killed, the usual feeding procedure was followed with respect to the quantity of feed offered and either ^{144}Ce or Cr_2O_3. All the calves were killed approximately 4 hours after feeding, and after being rendered unconscious by a blow to the head. After killing, the gastrointestinal tracts were tied off, removed, and divided into the reticulo-rumen, omasum, abomasum, 6 sections of the small intestine, cecum, and 2 sections of the large intestine. Contents of each section were weighed and mixed before sampling. Two 4-g samples of the ingesta from each segment were placed into plastic tubes for counting the ^{144}Ce in a γ-spectrometer (groups A and C). Chromic oxide in the ingesta was determined by the method of Brisson (14).

Samples of the ingesta were weighed into crucibles and dried 24 hours at 100°. After a dry weight was obtained the samples were ashed at 600°. The ash was dissolved in 6 N HCl and made up to a constant volume. Aliquots of the ashed samples were used for all mineral determinations.

Sodium and potassium concentrations in the ingesta were determined by flame photometer. Calcium and magnesium were determined by the complexometric titration method of Kamal (15) for the first 2 groups run (A and C) and by an atomic absorption spectrometer which became available for the third group (B). Nitrogen was determined by the Kjeldahl procedure according to AOAC (16).

RESULTS AND DISCUSSION

The daily intake of each calf was held constant for 8 days before killing. The average daily intakes of the respective rations were: semipurified, 2.0 kg; concentrate, 2.8 kg; and concentrate + hay, 2.1 kg concentrate and 1.2 kg hay. Water was offered ad libitum and no measurement was made of the daily intake.

[3] Grams per 100 g of salt mix: Mn, 0.025; Fe, 0.100; S, 0.050; Cu, 0.033; Co, 0.015; Zn, 0.008; and I, 0.007.

The average percentages of the total dry matter in the tract that was present in the rumen were: semipurified 70%, concentrate 65% and concentrate + hay 58%. These values reflect the differences in the total dry-matter content in the tract as a result of differences in intake, rate of passage and digestibility of the rations. The total dry matter in the tract at the time of killing averaged 1.0, 3.5 and 5.3 kg for groups A, B and C, respectively. The dry-matter digestibility in the rumen of calves fed the semipurified ration was 60% compared with 10% for the calves on the other 2 rations. Dry-matter digestibility was calculated by marker ratio techniques. It is probable that the semipurified ration would be highly fermentable in the rumen and the quantity of material entering and leaving the omasum would be less. The semipurified ration would promote fermentative digestion in the rumen at the expense of hydrolytic digestion in the intestine. No significant amount of ingesta was present in the omasum of the calves fed the semipurified ration. Many aspects of the physiological mechanisms involved in the functioning of the omasum remain to be explored.

Briggs [4] reported that approximately 7% of the volume entering the omasum could not be accounted for in the material leaving the organ. This value does not support the contention that approximately 50% of the water entering the omasum is absorbed. However, Briggs [5] used polyethylene glycol as an unabsorbed marker which is water-soluble. In this study by comparing the concentration of the unabsorbed marker in the ruminal contents with the concentration in the omasal contents a value of approximately 60% of the water in the ruminal contents could not be accounted for in the omasal contents. This does not necessarily indicate that 60% of the water entering the omasum is absorbed. Rather, it may indicate that the material entering the omasum is compacted and the soluble portion passed into the abomasum. If this occurs to a great extent a water-soluble marker would underestimate the actual water absorption if measured for a relatively short time period. Conversely, if the unabsorbed marker traveled with or adsorbed onto the undigested portion of the ingesta, it would overestimate the actual absorption of water or other constituents by the omasum following the described procedure. A high percentage of ^{144}Ce adsorbs onto the undigested residues (17). The significantly lower ($P < 0.05$) dry-matter content already present in the rumen of the calves fed the semipurified ration and the higher digestibility of the ration account for the lower dry matter content in the small intestine and cecum of this group compared with the other 2 groups (table 2).

The calcium concentrations in the intestinal contents are given in table 3. All values in this and subsequent tables are expressed as milligrams per gram of feed ingested. These values are obtained by determining the quantity of the unabsorbed marker present in a gram of feed that was fed and comparing the same quantity of marker in the ingesta to the determined concentration. This can be expressed by the following equation:

$$\frac{\text{Marker (\% of daily oral intake) in 1 g feed}}{\text{Marker (\% of daily oral intake) in 1 g ingesta}} \times \text{mg nutrient in 1 g ingesta} = \text{mg nutrient/g feed ingested.}$$

Net secretion has occurred if these values are greater than the amount originally contained in the feed. Conversely, if the values are less than the amount in the feed, net absorption has occurred. For analyses, the number of small intestine segments was reduced to three. The distribution of Ca in the intestinal contents compares favorably with the values reported by Chandler and Cragle (4). However, it appears unlikely that calcium is absorbed from the rumen in the quantity that is indicated by the calves that received the semipurified and concentrate rations. Storry (18) could not demonstrate a net loss of Ca or Mg ions from the rumen even with solutions that were sufficient to overcome potential and concentration gradients. The slight gain in the calcium content in the rumen of the calves that received the concentrate + hay ration probably is due to an influx of calcium into the rumen via the saliva (19). No net secretion or absorption of Ca occurred in the omasum. The abomasum

[4] Briggs, P. K. 1961 The influence of lipids on the utilisation of dietary nitrogen by the ruminant. Thesis, Aberdeen University, England.
[5] See footnote 4.

TABLE 2

Dry matter concentrations in intestinal contents of calves fed semipurified, concentrate and concentrate + hay rations

	Semipurified	Concentrate	Concentrate + hay
	%	%	%
Rumen	6.9 ± 1.1 [1]	18.5 ± 3.8	17.6 ± 0.9
Omasum		30.9 ± 1.2	25.1 ± 1.5
Abomasum	10.8 ± 4.4	21.2 ± 2.9	13.5 ± 5.4
Small intestine, section 1	7.8 ± 1.1	11.0 ± 2.8	11.3 ± 2.8
Small intestine, section 2	6.2 ± 1.7	9.7 ± 0.7	9.6 ± 0.3
Small intestine, section 3	5.8 ± 1.1	8.7 ± 2.1	7.3 ± 0.8
Small intestine, section 4	5.3 ± 0.4	8.1 ± 1.3	6.6 ± 0.7
Small intestine, section 5	4.6 ± 1.9	8.0 ± 0.6	6.9 ± 1.2
Small intestine, section 6	5.0 ± 2.0	11.2 ± 2.4	10.0 ± 0.5
Cecum	9.4 ± 2.7	17.5 ± 1.4	14.1 ± 1.1
Large intestine, section 1	14.4 ± 4.2	19.2 ± 0.1	15.0 ± 1.7
Large intestine, section 2	21.9 ± 1.4	22.9 ± 1.4	18.7 ± 2.2

[1] Mean ± SD.

TABLE 3

Concentration of Ca in intestinal contents of calves fed a semipurified, concentrate or concentrate + hay ration

	Semipurified	Concentrate	Concentrate + hay
		mg/g feed ingested	
Rumen	3.8 ± 0.7 [1]	4.4 ± 0.8	8.0 ± 3.0
Omasum		3.0 ± 0.6	7.8 ± 1.0
Abomasum	3.6 ± 0.9	8.2 ± 2.3	10.4 ± 1.4
Small intestine, section 1	4.0 ± 0.7	3.9 ± 1.7	5.1 ± 1.5
Small intestine, section 2	3.8 ± 1.3	2.9 ± 0.9	5.7 ± 1.5
Small intestine, section 3	3.1 ± 0.6	2.8 ± 0.6	5.6 ± 1.0
Cecum	2.8 ± 0.8	2.1 ± 0.5	4.8 ± 1.4
Large intestine, section 1	2.5 ± 0.4	1.9 ± 0.6	4.5 ± 1.0
Large intestine, section 2	2.7 ± 0.4	1.8 ± 0.3	4.5 ± 1.0
Ca in feed, mg/g	5.1	6.7	7.5
Avg daily intake, g	9.6	19.0	20.3

[1] All intestinal concentrations = mean ± SD.

presented a somewhat different picture. In some calves a net secretion of Ca was evident and in others a net absorption was indicated. Yang and Thomas (10) reported that in 14 of 24 calves more Ca was absorbed than secreted in the abomasum. It is known that practically all of the Ca in the abomasal contents is ultrafiltrable because of the hydrogen ion concentration (18, 19). Therefore, the best possible milieu exists in the abomasal contents for absorption of Ca but under certain conditions net secretion is indicated. Phillipson and Storry (20) could not demonstrate a net loss of Ca from the duodenum where the environment in the ingesta would be similar to that in the abomasum.

The major region of Ca absorption was the small intestines in all the calves. Very little change in Ca concentration occurred in the cecum and large intestine. The Ca concentration in the contents from the lower small intestines and large intestine of the calves receiving the concentrate + hay ration was significantly higher ($P < 0.05$) than for the other 2 groups. A partial explanation for this may be that a larger portion of the calcium in the contents from these locations was in a complexed form. Young milk-fed calves are known to be very efficient in their ability to absorb Ca (13).

The intestinal Mg concentrations are given in table 4. No change occurred in the ruminal Mg concentration in relation to the feed ingested. While it is possible that the omasum may be permeable to Mg (21), it is unlikely that the net absorption from this organ was as great as indicated in group C. It is probable that the marker was adsorbed to the undigested residues and consequently overestimated absorption

from the omasum. An apparent net secretion of Mg occurred in the upper small intestine of all calves. Storry (19) reported that bile and pancreatic juice contained appreciable quantities of both calcium and magnesium. The quantity of calcium and magnesium entering the small intestine is of such magnitude that net absorption of these elements cannot be detected in passage through the upper small intestine. Net absorption occurred in passage through the small intestine with little change thereafter. This is in contrast with the work of Smith (22, 23) in which it was reported that the absorption of Mg in calves occurred principally in the large intestine. However, these data are in agreement with the results of Stewart and Moodie (21) and Field (24) that the major region of Mg absorption is the small intestine. The apparent absorption of Mg with all the rations was 35%. Smith (8) reported Mg absorption of 30 to 40% in milk-fed calves. In later work, Smith (9) observed that in calves fitted with ileal reentrant fistulas, Mg had a quantitative effect upon the ileal water emergence, suggesting that it was present in a soluble or ionic form. If it is assumed that the calves in this study behaved similarly, it appears that other factors may be more important in Mg absorption than its presence in a form that can be readily absorbed. There were no apparent differences in the Mg concentration in the intestinal contents of the calves that received the different rations.

The concentration of Na in intestinal contents is given in table 5. There were no significant differences ($P < 0.05$) in the

TABLE 4

Concentration of Mg in intestinal contents of calves fed a semipurified, concentrate or concentrate + hay ration

	Semipurified	Concentrate	Concentrate + hay
		mg/g feed ingested	
Rumen	0.9 ± 0.4 [1]	2.7 ± 0.5	2.6 ± 0.1
Omasum		1.7 ± 0.4	1.6 ± 0.1
Abomasum	0.9 ± 0.4	1.7 ± 0.8	2.9 ± 0.1
Small intestine, section 1	3.0 ± 1.9	4.5 ± 1.6	3.3 ± 0.8
Small intestine, section 2	2.1 ± 1.5	3.1 ± 1.6	2.4 ± 0.3
Small intestine, section 3	1.5 ± 0.6	2.9 ± 0.9	2.2 ± 0.5
Cecum	0.8 ± 0.4	2.1 ± 0.5	1.7 ± 0.1
Large intestine, section 1	0.8 ± 0.3	1.9 ± 0.4	1.6 ± 0.2
Large intestine, section 2	0.6 ± 0.2	1.7 ± 0.1	1.7 ± 0.4
Mg in feed, mg/g	0.9	2.5	2.6
Avg daily intake, g	1.6	7.0	7.0

[1] All intestinal concentrations = mean ± SD.

TABLE 5

Concentration of Na in intestinal contents from calves fed a semipurified, concentrate or concentrate + hay ration

	Semipurified	Concentrate	Concentrate + hay
		mg/g feed ingested	
Rumen	15.9 ± 3.3 [1]	10.5 ± 3.3	12.3 ± 3.7
Omasum		2.0 ± 0.1	3.6 ± 1.2
Abomasum	15.5 ± 4.8	5.3 ± 3.0	10.3 ± 3.7
Small intestine, section 1	56.5 ± 19.1	41.1 ± 20.0	34.9 ± 15.2
Small intestine, section 2	39.2 ± 5.1	26.3 ± 10.0	28.0 ± 9.7
Small intestine, section 3	21.3 ± 15.3	13.0 ± 6.0	14.0 ± 5.0
Cecum	4.8 ± 2.9	2.2 ± 0.7	3.7 ± 0.4
Large intestine, section 1	2.3 ± 2.3	1.1 ± 0.4	2.3 ± 0.6
Large intestine, section 2	0.7 ± 0.3	0.6 ± 0.3	0.9 ± 0.4
Na in feed, mg/g	12.4	3.0	3.9
Avg daily intake, g	24.5	8.4	9.9

[1] All intestinal concentrations = mean ± SD.

concentration of Na in the rumen of the calves fed the different rations when expressed as milligrams per gram of feed ingested. However, the total ruminal Na load was greater in the calves that received the concentrate + hay ration. The average ruminal Na load was 28, 16 and 40 g for groups A, B and C, respectively. This was mostly due to the amount of ingesta in the rumen indicating rapid passage for the concentrate ration. This contention is supported by the amount of the unabsorbed markers in the rumen. The average amount of Cr_2O_3 or ^{144}Ce in the rumen (expressed as % of daily dose) was 99, 73 and 103% for groups A, B and C, respectively. The greater influx of Na into the rumen of the calves receiving the concentrate and concentrate + hay rations was probably a consequence of increased rumination on these rations. It is known that Na is absorbed from the rumen (25, 26), but the quantity of Na in the saliva entering the rumen was greater than the absorption from this organ. Sperber and Hyden (26) reported that Na was absorbed from the rumen against a concentration gradient whereas Parthasarathy and Phillipson (25) did not demonstrate such Na transport.

There was a large, variable, net secretion of Na into the upper small intestine. Normally other factors are probably more important in determining the volume and composition of these secretions than the daily intake of Na. Absorption occurred throughout the remainder of the tract. In all calves the ingesta reached the cecum before any net absorption occurred (on the basis of 3 segments of small intestine). Smith (8) reported that 40% of the Na intake was absorbed from ingesta at the lower end of the small intestine. The percentage of intake of Na absorbed was 94, 80 and 78% for groups A, B and C ($P < 0.05$, $A > B + C$). The absorption of Na from the small intestine accounted for a much greater percentage of the total Na flux from the gut to the blood than the absorption that occurred in the large intestine. Minimal values of secretion or absorption can be obtained by determining the quantity of Na that would have to be absorbed or secreted to effect changes from one segment to an adjacent segment.

An average estimate for all the calves is that 133 g of Na/day was secreted into the upper intestine. This value ranged from 92 to 158 g which is equal to 55 to 60% of the total body Na; however, the actual percentage would be less than this due to recycling. The absorption of Na from the small intestine accounted for 87% of the total Na absorbed from the lower gut. Only 13% of the total Na absorbed from the lower gut was absorbed during passage through the cecum and large intestine. The average net secretion of Na into the gastrointestinal tract was 159 g (121–181) per day. The average net absorption of Na from the gastrointestinal tract was 168 g (128–190) per day. It is evident that any malady which adversely affects the absorption of Na from the cecum and large intestine may disrupt the acid-base equilibria of the animal. If such a phenomenon occurred, the condition may be intensified by sequestration of Na into the gut and the resultant negative Na balance.

The K concentrations in the intestinal contents are shown in table 6. No appreciable changes occurred in the ruminal concentration of K per gram of feed ingested of the calves that received the concentrate and concentrate + hay rations. However, an average of 40% of the ingested K was absorbed from the rumen of the calves that received the semipurified ration. Sperber and Hyden (26) reported that the ruminal K concentration was 5 times that in the plasma, suggesting that this element passively diffuses across the rumen epithelium. The apparent net absorption of K from the rumen of the calves on the semipurified ration is probably due to the greater concentration gradient resulting from the high K concentration in the feed. By implementing the same formula for K as used previously for Na, minimal net absorption and secretion values can be obtained. In the upper portion of the small intestine it is estimated that there was an average net secretion of 36 g (15–45) of K/day. It is also estimated that an average of 36 g (15–55 g) of K was absorbed from the small intestine daily and that an average of 4 g (2.5–5.0) was absorbed from the cecum and large intestine daily. No differences were observed that could be

attributed to the type of ration. The concentration of K in the gut appeared to be the least affected by its concentration in the feed consumed than any of the other minerals that were measured. This was probably due to the translocation of K from the rumen to the urine. The greater K intake of the calves that received the semipurified ration was excreted in the urine. It is noteworthy that the K concentration in the contents from the upper small intestine of group A did not attain the level initially present in the ration even with the rather large secretion of K that occurred in this region. The percentage of the K intake absorbed was 98, 75 and 80% for groups A, B and C, respectively. The lower values for both Na and K absorption in groups B and C may be a consequence of the higher fiber content of these rations which impaired absorption in the large intestine. It is possible that the physical bulk of the undigested residues shielded the Na or K from coming into contact with absorptive surfaces in this area (11).

The total nitrogen concentration in the intestinal contents is shown in table 7. There was a disappearance of N from the rumen of the calves that received the semipurified ration. No change in ruminal concentration occurred in groups B and C. A large, highly variable, secretion of N occurred in the upper portion of the small intestine. Absorption of N occurred throughout the small intestine in all calves with no further change evident in large intestine. These results probably are not very meaningful since the origin of the nitrogen is not determined. The problems involved in partitioning the N with respect

TABLE 6

Concentration of K in intestinal contents from calves fed a semipurified, concentrate or concentrate + hay ration

	Semipurified	Concentrate	Concentrate + hay
		mg/g feed ingested	
Rumen	12.4 ± 6.7 [1]	4.2 ± 0.9	6.5 ± 1.8
Omasum		1.7 ± 0.3	2.5 ± 0.5
Abomasum	8.9 ± 4.4	3.0 ± 0.9	3.4 ± 0.3
Small intestine, section 1	16.4 ± 5.2	17.9 ± 8.7	10.8 ± 6.3
Small intestine, section 2	9.2 ± 5.8	7.5 ± 2.3	6.7 ± 2.3
Small intestine, section 3	3.2 ± 1.3	5.7 ± 3.7	3.0 ± 0.7
Cecum	1.3 ± 0.4	2.0 ± 0.3	2.5 ± 0.5
Large intestine, section 1	0.6 ± 0.2	1.6 ± 0.2	1.8 ± 0.2
Large intestine, section 2	0.3 ± 0.1	1.1 ± 0.5	1.0 ± 0.5
K in feed, mg/g	20.2	4.2	7.8
Avg daily intake, g	39.0	11.8	25.8

[1] All intestinal concentrations = mean ± SD.

TABLE 7

Concentration of nitrogen in intestinal contents of calves fed a semipurified concentrate or concentrate + hay ration

	Semipurified	Concentrate	Concentrate + hay
		mg/g feed ingested	
Rumen	26.9 ± 3.5 [1]	29.1 ± 9.1	22.5 ± 1.6
Omasum		31.1 ± 4.8	18.0 ± 2.5
Abomasum	35.1 ± 13.4	36.0 ± 15.5	23.5 ± 7.0
Small intestine, section 1	133.9 ± 46.8	164.6 ± 32.8	94.1 ± 14.6
Small intestine, section 2	66.4 ± 30.6	76.5 ± 19.1	47.2 ± 17.8
Small intestine, section 3	19.6 ± 7.2	34.4 ± 24.5	13.1 ± 2.5
Cecum	11.4 ± 5.3	12.0 ± 1.3	8.6 ± 0.2
Large intestine, section 1	7.8 ± 1.0	11.9 ± 1.6	8.4 ± 1.7
Large intestine, section 2	7.1 ± 1.8	11.1 ± 0.6	8.4 ± 0.3
N in feed, mg/g	6.9	20.1	20.4
Avg daily intake, g	1.2	57.1	68.1

[1] All intestinal concentrations = mean ± SD.

to origin are immense. It is known that the total nitrogen in the ingesta is contributed by 5 general sources: 1) soluble protein from secretions and feed; 2) cellular proliferation of the intestinal mucosa; 3) desquamated cells in the pancreatic secretion; 4) nonprotein nitrogen; and 5) bacteria. Until simple techniques are available to determine the contribution from each source, studies on the intestinal N concentrations leave much to be desired.

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Transmural Movements of Zinc, Manganese, Cadmium and Mercury by Rat Small Intestine

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ABSTRACT To elucidate some features of the mechanism of distribution and localization of trace metals in soft mammalian tissues, we studied the simultaneous uptake and transport patterns of 2 essential metals, Zn and Mn, and 2 nonessential metals, Cd and Hg. The radioisotopes of the metals and loops of rat small intestine were used in a new in vitro perfusion method. The characteristic rates of metal uptake and metal transport varied markedly. During the first hour of perfusion, very small amounts of Zn, Hg or Cd were transported across the rat intestinal wall, whereas Mn was transported to an appreciable extent. Except for Mn, when the tissue uptake of a metal was high, its accumulation in the serosal solution was proportionately slow. The relative uptake-to-transport molar ratios for Zn, Hg, Cd and Mn were approximately 20:12:6:1, respectively. The uptake or transport of a metal was strikingly enhanced or depressed by the presence of a second metal particularly when the initial concentration of the second metal exceeded the concentration of the first. The effects of each of the chemical agents, L-ascorbate, dehydroascorbate, phlorhizin, iodoacetate and EDTA on the simultaneous uptake and transport of each of these 4 metals were measured. Zinc, Cd and Mn uptakes were depressed by L-ascorbate, dehydroascorbate or EDTA, whereas their transports were enhanced, except for Mn transport which was unaffected by L-ascorbate. Iodoacetate depressed Zn and Hg transports but markedly enhanced the uptakes of Zn, Hg, and Cd. Phlorhizin enhanced the uptake and depressed the transport of Cd nearly 3-fold. Both Zn uptake and transport were enhanced by phlorhizin. The transmural passages of this tetrad of metals Zn, Mn, Cd, and Hg are not dependent on the expenditure of metabolic energy, are not unidirectional, and are not carried against a concentration difference. Competition among these metals for uptake and transport appears to be primarily a competition for the common available uptake sites. Transport is controlled to a lesser degree by the diffusibility characteristics of the ions of the particular metal.

Advances in understanding the nature of the mechanisms involved in the absorption of divalent trace metals by cells and tissues have been slow. Relatively little is known about the characteristics of the movements of these metals whereby their tissue concentrations are regulated. That there is some control mechanism operative at the cell membrane level, at least for the essential metals, is suggested by their selective tissue localization and their variability in human tissues [3] which have been examined (1–5).

In a previous communication, we reported on the uptake of divalent Zn, Mn, Cd and Hg by intact strips of rat intestine (6). In the present study, with a newly developed in vitro perfusion method adapted for the use of the radioisotopes of the metals, we present evidence for the characteristic uptake and transport patterns of Zn, Mn, Cd and Hg by segments of rat small intestine. The uptake by the intestinal wall of the rat and transport across it were simultaneously measured. Observations on the effects of various pharmacologic agents on metal uptake and transport as well as mutual competition for transmural passage between pairs of these metals are also presented.

METHODS AND MATERIALS

Perfusion and counting technique. Normal adult rats of the Wistar strain of both sexes (250–350 g), fed a commercial lab-

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[3] Harding-Barlow, I. 1961 Studies on the trace element content of human tissue. Ph.D. Dissertation, The University of Capetown, Capetown, South Africa.

oratory ration [4] and tap water ad libitum, were fasted 48 hours before decapitation. Two adjacent segments of the small intestine, each 12.7 cm, from a section 10 to 15 cm distal to the duodenum, were excised, everted, and washed with ice-cold oxygenated physiologic saline. These segments were mounted on duplicate perfusion apparatus and perfuson started at once. The perfusion medium consisted of 40 ml of Ca-free Krebs-Ringer solution, 10 mM in Tris buffer (pH 7.4) and 5 mM in glucose for the mucosal aspect of the intestinal segment and 10 ml of the same solution for the serosal aspect (7, 8). In addition, the mucosal solution contained labeled metal ions (10^{-4} M), the uptake and transport of which were being measured. For studies involving accumulation against a concentration difference, both aspects of the intestine contained aliquots of the same metal solution. All perfusions were carried out in pairs, control and unknown, at 37° and in an atmosphere of 100% O_2. Samples of mucosal and serosal solutions (0.3 ml) were removed at hourly intervals during 5 hours of perfusion. The transit of a metal across the intestinal wall was investigated by measuring the concentration of the particular metal ions appearing in the solution bathing the mucosal or serosal aspect of the gut wall when the metal ions were initially introduced into the solution bathing the opposite surface. This transit will be alluded to here as transport or transmural passage. At the termination of the experiment, the 12.7-cm segment of intestine was removed, washed on both sides with a stream of ice-cold physiologic saline, and its metal content measured. This tissue metal content is designated as tissue metal uptake or retention.

A Nuclear-Chicago radiation analyzer and scaler was used to measure gamma-emission. All readings were corrected for background. Unless otherwise indicated, all measurements of metal uptake and transport were performed on at least three different segments of intestine each from a different rat, and all results cited are the mean of such triplicate determinations. The variability within triplicate analyses of uptakes for the same metal was less than ± 10%; the variability for transports was within ± 6%. All results were expressed as millimicrogram-atoms of metal/12.7 cm of intestinal segment.

The perfusion apparatus (fig. 1) used in these studies consists essentially of two separate systems, a mucosal (40 ml) and a serosal (10 ml) circuit which communicate with each other only through the intestinal segment which can be everted and mounted or mounted without eversion thus permitting the interchange of volumes encountering the 2 surfaces. The segment which is tied to the apparatus with surgical silk assumes the shape of a loop and is normally relaxed and free to move, simulating a more normal physiologic condition for this tissue than is obtained with other in vitro methods. A pair of such units permits the simultaneous running of control and unknown with adjacent seg-

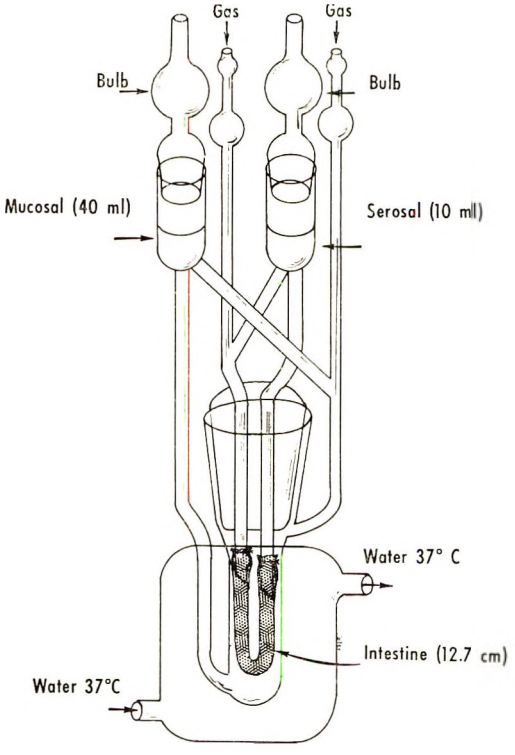

Fig. 1 Glass perfusion apparatus used for the continual measurement of substances transported across the rat intestinal wall and their uptake by the tissue itself. A pair of such units permits the simultaneous running of control and unknown with contiguous segments of small intestine.

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

ments of intestine. Small volumes of fluid required make radioisotopic studies feasible. Both circuits are gassed with 100% O_2 which also provides the lift to circulate the solutions. The funnels provide the means for sampling or introducing other substances without interruption of perfusion. The outer jacket through which preheated water from a reservoir is circulated by means of a Porta-Temp pump [5] maintains the desired temperature. The bulbs attached to the sampling funnels help control the foaming. The apparatus with ground glass joints is constructed entirely of Pyrex glass which facilitates both mounting and cleaning operations. With this perfusion apparatus, we have measured the rates at which metal ions are transported across the intestinal wall of the rat and the total amounts of the metals taken up or retained by the intestinal segment itself at the termination of perfusion.

Compounds and conventions used. Analytical reagent grade chlorides of Zn and Mn and the nitrates of Cd and Hg were used. The radioisotopes of the metals were: ^{54}Mn, carrier-free, obtained from the New England Corporation; and ^{65}Zn, ^{203}Hg and ^{115m}Cd, obtained from Oak Ridge National Laboratory. Uniformly labeled ^{14}C-D-glucose was obtained from the International Chemical Nuclear Corporation.

RESULTS

Active transport of glucose. Before our studies with metals, the functional adequacy of the perfusion apparatus was tested with the use of uniformly labeled ^{14}C-D-glucose. Initially, the same concentration of labeled glucose (5×10^{-3} M) was placed on both sides of the gut wall. Hourly samples were taken from both solutions. The concentration of glucose on the mucosal side decreased, and the concentration of glucose on the serosal side increased during a period of 3 hours of perfusion. A total of 570 mμmoles of glucose/12.7 cm intestine/3 hours was transported from the mucosal to the serosal solution against an increasing difference of concentration of glucose.

Relative rates of uptake and transport of the metals. Individual solutions containing Mn, Cd, Hg or Zn ions (10^{-4} M) were introduced to the mucosal aspect of a segment of rat intestine, and the hourly rate of accumulation of each metal in the solution bathing the serosal aspect was measured. Very small amounts of Cd, Hg, and Zn were transported within the first hour of perfusion, whereas Mn was transported relatively rapidly. During 5 hours of perfusion (9) of a 12.7-cm segment of rat intestine, under aerobic conditions, appreciable amounts of each metal appeared in the respective serosal solution. The order of the relative rates of increase in concentration of these metals in the serosal solution, that is, rates of transmural passage, was: Mn > Cd > Hg > Zn (fig. 2).

The order of the amounts of metal taken up or retained by the same segment of intestine perfused for the same length of time, that is, tissue metal uptakes, was: Zn > Hg > Cd > Mn. This order is in agreement with our previous report on metal uptakes by the intact strip method (6) and is opposite to that of the order of transports of the metals across the gut wall or their rates of accumulation in the serosal solution (table 1).

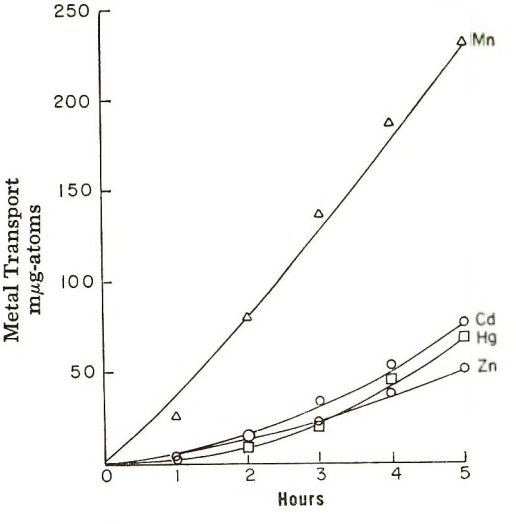

Fig. 2 Comparison of the transport rates of Mn, Cd, Hg, and Zn. Rat intestinal segments (12.7 cm) were everted and perfused. The initial mucosal concentration of each metal was 10^{-4} M, initial serosal concentration O. The perfusion medium (pH 7.4) (mucosal 40 ml, serosal 10 ml) consisted of Ca-free Krebs-Ringer solution with Tris buffer (10^{-2} M) and glucose (5×10^{-3} M). All perfusions were conducted at 37° and gassed with 100% O_2.

[5] Precision Scientific Company, Chicago.

TABLE 1

Effect of pharmacologic agents [1] *on intestinal metal uptake and metal transport*

	Mn		Cd		Hg		Zn	
	Uptake	Transport	Uptake	Transport	Uptake	Transport	Uptake	Transport
	mμg-atoms/5 hr/12.7 cm rat intestine							
Control [2]	240	229	459	75	831	68	959	49
Ascorbate	65	224	104	502	—	—	158	172
Dehydro-ascorbate	47	405	114	387	597	10	159	290
Phlorhizin	355	211	1203	22	550	89	1134	216
Iodoacetate	308	314	1014	69	1146	19	1124	3
EDTA	52	619	51	283	568	1	84	467

[1] Each metal tested was initially at 10^{-4} M. The concentration of the pharmacologic agents, ascorbate or dehydroascorbate was 10^{-2} M and that of phlorhizin, iodoacetate and EDTA each was 10^{-3} M.
[2] Variability of individual values was within ±10% of the mean for metal uptakes and within ± 6% for metal transports.

Effects of pharmacologic agents on the uptake and transport of the metals. The effects of EDTA, dehydroascorbate,[6] ascorbate,[7] iodoacetate and phlorhizin on the transports of Mn, Zn, Cd or Hg across the rat intestinal wall were investigated when these were initially present only on the mucosal side of the gut wall.

Mn transport was greatly enhanced in the presence of EDTA and, to a lesser degree, in the presence of dehydroascorbate and iodoacetate. Ascorbate and phlorhizin show little or no effect on Mn transport (fig. 3).

Zn transport was enhanced in the presence of EDTA, dehydroascorbate, phlorhizin and ascorbate, in the order cited, whereas iodoacetate depressed Zn transport (fig. 4).

Cd transport was enhanced to a greater extent by ascorbate than by dehydroascorbate or by EDTA. Iodoacetate showed no effect on Cd transport, but phlorhizin slightly depressed it (fig. 5).

Hg transport was depressed by dehydroascorbate, iodoacetate and markedly by EDTA. Within the first 2 hours of perfusion, phlorhizin showed no measurable effect on Hg transport, whereas enhancement of Hg transport began after the second hour (fig. 6).

After termination of perfusion, the segment of intestine was washed on both sides with cold physiologic saline and its metal content measured. A comparison of the effects of the same group of pharmacologic

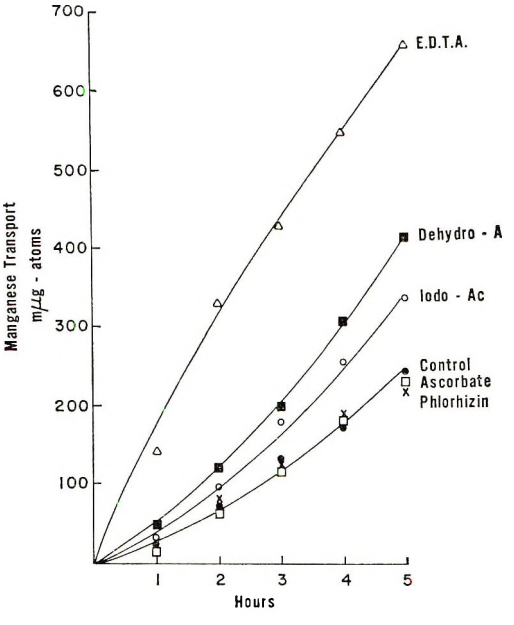

Fig. 3 The effect of chemical agents on Mn transport. Initial mucosal concentrations were: Mn, 10^{-4} M; ascorbate and dehydroascorbate (Dehydro-A) 10^{-2} M; phlorhizin, iodoacetate (Iodo-Ac) and ethylenediaminetetracetic acid (EDTA) 10^{-3} M. Initial serosal concentration for each substance was O. All other conditions of perfusion were as described in the legend for figure 2.

[6] L-Ascorbic Acid, C.P., Lot no. 540-400, Fischer Scientific Company, Fair Lawn, New Jersey.
[7] Dehydroascorbic Acid, Lot no. 63979F, K and K Laboratories Inc., Plainview, New York.

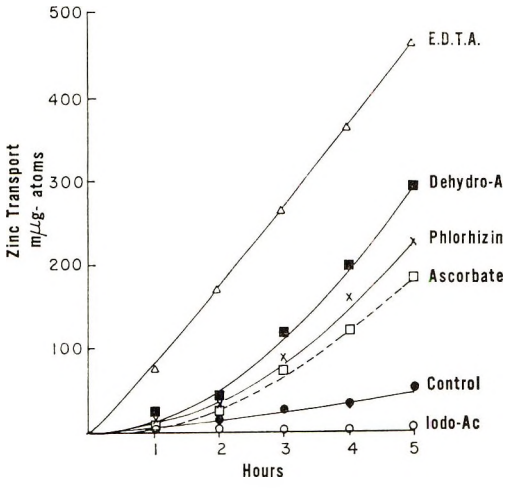

Fig. 4 The effect of pharmacologic agents on Zn transport. Initial mucosal concentration of Zn was 10^{-4} M. All other conditions of perfusion were as described in the legend for figure 3.

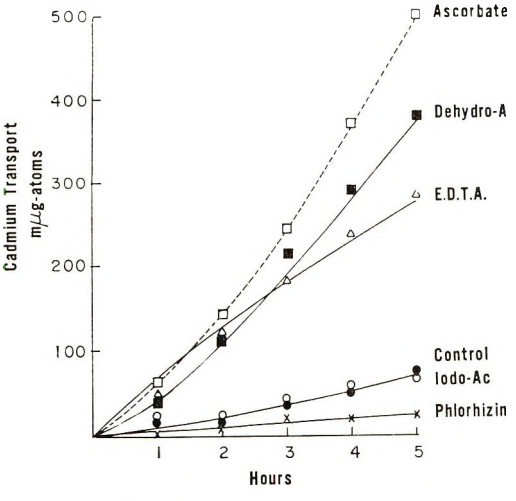

Fig. 5 The effects of pharmacologic agents on Cd transport. Initial mucosal concentration of Cd was 10^{-4} M. All other conditions of perfusion were as described in the legend for figure 3.

agents on the uptake and on the transport of these metals is presented in table 1. The action of ascorbate on Hg transport could not be studied because ascorbate rapidly reduces divalent mercury. With some exceptions, whenever the uptake of a metal was enhanced by the action of the pharmacologic agent added, its transport was correspondingly reduced and vice versa. In some instances, both uptake and transport appeared to be enhanced (phlorhizin on Zn) or depressed (EDTA and dehydroascorbate on Hg), although not to the same degree, and in others uptake increased or decreased while transport remained relatively little affected (phlorhizin on Mn or iodoacetate on Cd, and ascorbate on Mn).

Competitive effect of one metal on the transport of another. The transport of Zn, Mn, Cd and Hg each at a concentration of approximately 10^{-5} M was measured individually in the presence of one of the other three metal ions at an initial concentration at least tenfold greater. These results are presented in figure 7. Manganese transport is greatly enhanced by Cd and depressed by Hg. Cadmium transport is enhanced by Zn and Hg, and Hg transport is enhanced by Cd. Zinc transport is enhanced by Cd. The uptake of each of these metals was also measured in the presence of each of the others. Competitive uptake results obtained by this method were essentially in agreement with those obtained by the intact strip method previously reported (6).

Nature of transports of Zn, Mn, Cd, and Hg. Transport against a concentration difference was examined. For each metal, aliquots of a solution having the desired concentration of the metal were placed on both the mucosal and serosal sides of the gut wall and perfusion started. These results are presented in figures 8–11. For each metal tested, both serosal and

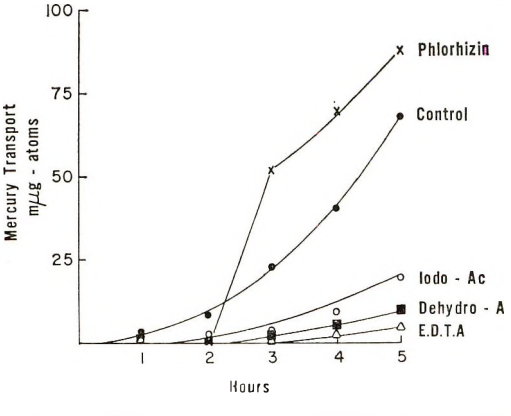

Fig. 6 The effect of chemical agents on Hg transport. Initial mucosal concentration of Hg was 10^{-4} M. All other conditions of perfusion were as described in the legend for figure 3.

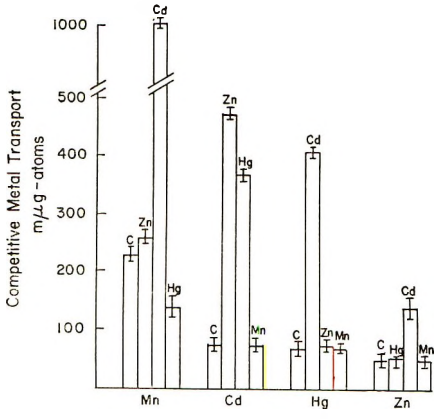

Fig. 7 The competitive effect of one metal on the transport of another. The initial mucosal concentration of each of the metals were: Mn, 10^{-5} M; Cd, 3.07×10^{-4} M; Hg, 3.48×10^{-5} M; and Zn, 1.26×10^{-5} M. The corresponding initial mucosal concentration of each metal Hg, Cd, or Mn for measurement of its effect on respective Zn, Mn or Hg transport was 5×10^{-4} M for Cd transport 2×10^{-3} M. All other conditions of perfusion were as described in the legend for figure 3. The symbol above each column designates the metal whose effect on the transport of the particular metal indicated on the X-axis is being measured. In each case, C indicates the control value.

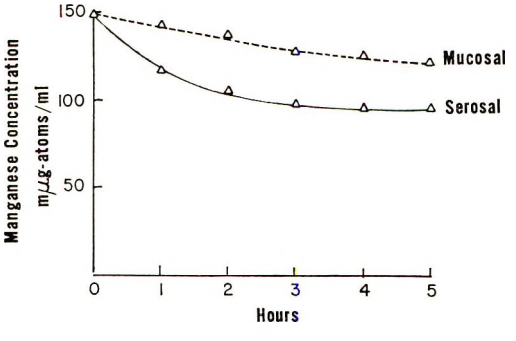

Fig. 8 Manganese transport against a concentration difference. Both initial mucosal and serosal Mn concentrations were the same (1.5×10^{-4} M). All other conditions of perfusion were as described in the legend for figure 3.

mucosal concentrations decreased rapidly within the first 2 hours of perfusion and appeared to reach equilibrium values during the ensuing 3 hours. No increase in concentration of metal for either the serosal or mucosal solutions was observed for any of the 4 metals, despite the fact that although initial concentration of the solutions on both aspects of the gut wall were the same, the total amounts of metal contained in the mucosal solutions were 4 times that of the serosal solutions. The order of the rate of decrease of the concentration of the solutions for each metal was in agreement with the order of metal uptakes reported previously (6).

Transport of the metals in the presence of 2,4-dinitrophenol (DNP) was carried out during 3 hours of perfusion. Unex-

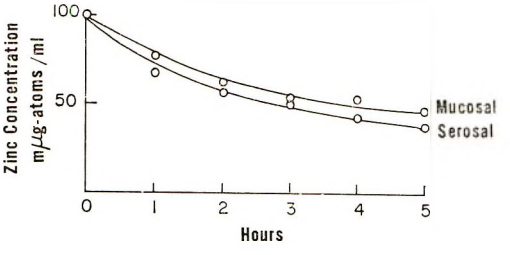

Fig. 9 Zinc transport against a concentration difference. Both initial mucosal and serosal Zn concentrations were the same (10^{-4} M). All other conditions of perfusion were as described in the legend for figure 3.

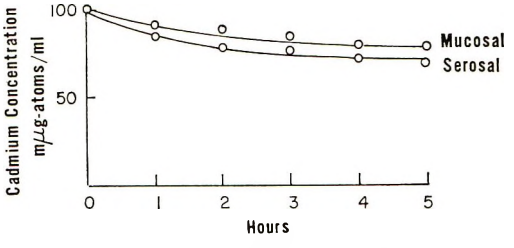

Fig. 10 Cadmium transport against a concentration difference. Both initial mucosal and serosal Cd concentrations were the same (10^{-4} M). All other conditions of perfusion were as described in the legend for figure 3.

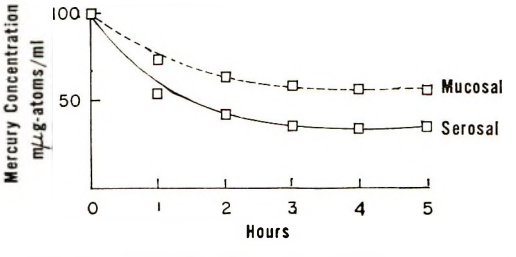

Fig. 11 Mercury transport against a concentration difference. Both initial mucosal and serosal Hg concentrations were the same (10^{-4} M). All other conditions of perfusion were as described in the legend for figure 3.

pectedly, the transport of each metal was (enhanced considerably in the presence of (10^{-4} M) DNP (table 2). During the first hour of perfusion, Mn transport was enhanced approximately sixfold and Cd transport threefold, whereas Zn and Hg transports were little affected by DNP. During the ensuing 2 hours of perfusion, all four metal transports were increased by action of DNP. Apparently, whatever the action of DNP, it is involved in the transport of these metals and not in their uptakes (6).

The rates of transport of each metal from the mucosal to the serosal side and vice versa were compared. In one case, the segment of intestine was everted and perfused thus initially having its mucosal side exposed to the metal ions, and in the other an adjacent segment was perfused without everting, the serosal side being initially exposed to the metal ions. The solutions bathing the respective mucosal and serosal sides of the gut wall were exactly the same in metal concentration and total amounts of metal present. The corresponding serosal and mucosal sides initially had no metal present. Under these conditions, Mn transport was equally rapid in either direction, whereas Cd, Hg and Zn transports were approximately 2 to 3 times greater from the serosal to the mucosal side than were their transports in the opposite direction. Simultaneous uptake measurements indicated approximately the same degree of metal uptake by gut tissue regardless of the direction of perfusion of the metal ions (table 3).

DISCUSSION

Despite their low concentrations in biological systems, the trace metals accumulate selectively in certain parts of the mammalian body (1–5, 10–15). To elucidate some features of this selective organ and tissue localization of the trace metals, we studied the simultaneous uptake and transport of a pair of essential metals, Zn and Mn, and a pair of nonessential metals, Cd and Hg. Segments of rat small intestine were perfused with the radioisotopes of the metals for several hours using a newly developed in vitro perfusion method. The uptakes measured by this method were in close agreement with those reported previously for the intact strip method (6). The transports or transmural passages were extremely slow for Zn and Hg, relatively more rapid for Cd, and most rapid for Mn. Only Mn was transported in appreciable amounts within the first hour of perfusion. With continued perfusion, their serosal concentrations increased such that for each metal a definite and characteristic pattern of transport became discernible.

TABLE 2

Effect of 2,4-dinitrophenol (DNP) on intestinal metal transport [1]

Perfusion	Mn		Cd		Zn		Hg	
	Control	DNP	Control	DNP	Control	DNP	Control	DNP
hours	*mμg-atoms/12.7 cm rat intestine*							
1	9.2	59.7	3.01	9.7	2.5	3.5	1.3	1.5
2	27.5	153.3	6.52	18.4	6.8	40.6	4.3	8.0
3	38.3	190.1	12.9	27.6	8.3	71.3	5.2	16.5

[1] The concentration of DNP (10^{-4} M) was the same for each metal transport. The concentration of the metals were: Mn, 10^{-4} M; Cd, 10^{-4} M; Zn, 1.15×10^{-4} M; and Hg, 0.8×10^{-4} M.

TABLE 3

Comparative directional transport rates of the metals

Direction [1]	Mn		Cd		Hg		Zn	
	Uptake	Transport	Uptake	Transport	Uptake	Transport	Uptake	Transport
	mμg-atoms/3 hr/12.7 cm rat intestine							
M → S	205	100	293	29	754	10	762	6
S → M	202	98	277	51	713	28	731	17

[1] Mucosal (M) to serosal (S) and vice versa.

The overall in vitro transmural passage of a metal by the rat intestinal wall appears to depend upon two separate steps. The first is the uptake and binding of the metal by extracellular and intracellular surfaces, and the second is the transport of the metal across cellular membranes and accumulation in the solution bathing the opposite surface of the wall. The degree of tissue retention of a metal appears to vary with the number and nature of the binding sites available to that metal; the rate of its transport appears to vary with the physicochemical nature of the particular metal ions (10, 16–24).

In the tetrad of metals examined here, when the tissue uptake of a metal by the rat intestinal wall was high, its transport across that wall was proportionately slow. The order of the rates of metal transports appears to be the reverse of the order of metal uptakes. The relative uptake to transport molar ratios for Zn, Hg, Cd, and Mn were: 20:12:6:1, respectively. Under the experimental conditions used here, the transport of a given metal was largely dependent upon the degree of affinity of the particular metal ions for binding sites available on the tissue, either on the outer or inner cellular surfaces, and the total capacity of the tissue for binding and retaining a given metal. In this connection, the presence of a number of classes of ligands of importance should not be overlooked, namely, sulfhydryl, carboxyl, phosphate and imidazole groups, which may be involved in the interaction of these metals with protein surfaces. In the presence of Tris buffer at pH 7.4 and 37°, explicit information about the nature and total number of binding sites in any one class or all classes of ligands present for a given metal ion is not available (18, 22). The question of precisely where a metal ion is bound to the protein surface or in what structural and stochiometric relationship, has not been resolved (23, 24). Nonetheless, our results indicate the overall comparative binding capacity of rat intestinal segments for the metals examined. Zinc and Hg accumulated in the gut wall to high levels, but almost no transmural passage of these metals was detectable within the first hour of perfusion. Tissue uptake of Mn was low whereas its transport was rapid. Cadmium uptake and transport occupied an intermediate position between Mn and Hg.

The uptake or transport of a given metal was affected by the presence of a second metal, particularly when the initial medium concentration of the second metal exceeded the concentration of the first. Since these metals compete with one another for uptake (6, 17, 18, 20), it was reasonable to expect that this competition for uptake would be reflected in their rates of transport. However, our results on competitive transports (fig. 7) cannot be explained solely on the basis of competitive tissue metal uptakes. The behavior of Zn and Cd toward Mn uptake is consistent with the concept of relative binding affinities (17–19); we cannot, however, readily account for the finding that Hg depresses Mn transport, whereas Cd increases it considerably. If Hg and Cd are bound to the same sites, should not their action on Mn be similar? The competition for transport between Zn and Cd, Hg and Cd appears to be well-defined. There is no apparent competition for transport beween Mn and Zn or Hg and Zn. On the other hand, Hg causes Cd transport to increase, and Cd causes Hg transport to increase. This appears to involve a common site and supports the view that essentially the competition between these 2 metals is one in binding to the available tissue sites and not in their transports.

Except for iodoacetate, the other 4 chemical agents used increased Zn transport. Iodoacetate depressed Zn transport but increased Zn uptake. This behavior of iodoacetate is in agreement with findings of Saltman and Boroughs (10) who reported enhancement of Zn uptake by liver slices in the presence of iodoacetate. In addition, our results indicate an enhancement of Hg, Cd and Mn uptake by iodoacetate (table 1). Phlorhizin (10^{-3} M) was used to ascertain whether the transports of these metals were dependent upon concomitant active uptake and transport of glucose. Our results are equivocal in this respect. Zinc and particularly Hg transports were enhanced by phlorhizin, whereas Cd transport was depressed and Mn transport apparently unaffected. Except for Hg, metal transports are enhanced by

ascorbate and dehydroascorbate, and metal uptakes are depressed. The enhancing action of ascorbate on metal transport appears to be unrelated to its powerful reducing action since dehydroascorbate has a similar effect. All four metal uptakes were substantially depressed in the presence of EDTA, and correspondingly their transports were enhanced except for Hg transport which was virtually nil in the presence of this metal complexing agent.

Our studies have shown a high degree of Zn and Hg accumulation in gut tissue with correspondingly slow movements of Zn and Hg ions across the gut wall. Some inhibition of Zn accumulation in liver slices by 2, 4-dinitrophenol (DNP) has been reported (10). The action of DNP on the transport of Zn, Hg, Cd and Mn, as indicated by our studies, is one of enhancement by several-fold for each metal. This finding would be contrary to expectations if we assumed the presence of an active process in the transport of these metals. However, at 10^{-4} M concentrations of DNP, the intestinal wall of the rat appears to undergo some changes in permeability which facilitate the transmural passage of these metals. Tidball has reported that Mg and Ca, loosely bound in the structure of the intestinal membrane of the rat, regulate the aqueous permability of the intestinal epithelium. The action of DNP may well be involved in these changes of membrane permeability (25).

The operation of an active, vitamin D-dependent, transport mechanism in the proximal small intestines of rabbits, rats, and guinea pigs, specific for Ca^{2+} and Mg^{2+} as contrasted with Sr^{2+} and Ba^{2+}, has been described by Schachter and Rosen (26). Competition between Ca^{2+}, Sr^{2+}, and Mg^{2+} for absorption in the isolated rat intestine incubated in Tris buffer medium of pH 7.4 was reported by Hendrix et al. (27). For none of the metals studied here was there a net increase against an apparent concentration difference. When the initial concentration of a metal was the same on both sides of the gut wall, the concentration of both sides decreased gradually as the metal was taken up by the tissue from either side of the gut wall simultaneously. Moreover, the transports of the metals were not unidirectional as is the case with active transport of sugars. For Mn transport, the rate of transport was about the same from either direction; for Cd, Hg, or Zn, transport rates were two- to threefold higher in the direction of serosal to mucosal side.

These studies appear to indicate that the transport of Zn, Mn, Cd or Hg is not dependent on expenditure of metabolic energy directly derived from oxidative processes, not unidirectional, and not transported against a concentration difference. Their uptakes and transports are influenced by the presence of each of the chemical agents used and, more importantly, competition among these metals for uptake and transport appears to be primarily a competition for the available uptake sites (28). Transport appears to be controlled to a lesser degree by the diffusibility characteristics of the ions of a particular metal.

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Alterations in the Activities of Several Rat Liver Enzymes at Various Times after Initiation of a High Protein Regimen [1]

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ABSTRACT The purpose of these experiments was to investigate the time course of increase of several rat liver enzymes following the feeding of a high protein diet (90% casein and carbohydrate-free). Male Sprague-Dawley rats, 6–8 weeks old and weighing 150–200 g were fed a 90% glucose, protein-free diet for 4 days in order to obtain maximal responses in activity after the dietary change. The animals were killed and liver enzyme activity was determined at 0, 1, 2, 3, 4 and 7 days after the rats were offered the high protein diet. All the enzymes studied, with the possible exception of phosphorylase, were increased by the high protein diet. Two patterns of increases in enzyme activity were observed. A number of enzymes including glucose 6-phosphatase, L-α-glycerophosphate dehydrogenase, pyruvate kinase, malic enzyme and tyrosine-α-ketoglutaric transaminase reached a maximum activity within 48 hours or earlier after the change in diet and either remained nearly constant or declined with time. The activities of another group of enzymes including fructose 1,6-diphosphatase, glucose 6-phosphate dehydrogenase, glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase and serine dehydrase rose sharply on the second and third day after the dietary change and the time course of increase appeared to approximate a sigmoidal curve.

It has been suggested that there may be two major mechanisms by which the amount of an enzyme is increased in higher animals (1,2): (a) by increasing its rate of synthesis and (b) by decreasing its rate of catabolism. The first mechanism, if it requires messenger-RNA synthesis, is susceptible to actinomycin D inhibition (2). The second mechanism (which does not require messenger-RNA synthesis and is, therefore, not susceptible to actinomycin inhibition) requires the presence of a stabilizing agent. An increase in the amount of enzyme by this latter mechanism is expected to be inhibited by puromycin (2) since in this mechanism continued synthesis of the enzyme is still essential. This characteristic of mechanism (b) distinguishes it from a conversion of inactive to active enzyme, since such a conversion would not be expected to be sensitive to puromycin inhibition.

Induction of an enzyme by mechanism (a) should produce a parabolic curve of activity versus time, while if the induction takes place by mechanism (b), enzyme activity should increase linearly. The induction of liver tryptophan pyrrolase by cortisol was shown by Schimke et al. (3) to be brought about by mechanism (a). Treatment with tryptophan in vivo, on the other hand, increased tryptophan pyrrolase activity by mechanism (b) (3).

It has been shown that the activity of tyrosine-α-ketoglutaric transaminase (4), serine dehydrase (5, 6), glutamic-pyruvic transaminase (4, 7, 8), glutamic-oxalacetic transaminase (4, 7), glucose 6-phosphatase (9, 10), fructose 1,6-diphosphatase (11), glucose 6-phosphate dehydrogenase (7), and kidney glutaminase (12), can be increased by feeding a high protein diet while the activity of pyruvate kinase is decreased (13, 14). The activity of malic enzyme is increased by a diet rich in glucose or fructose (15, 16).

These findings prompted us to undertake a study of the patterns in changes of enzyme activity with time after feeding a high protein diet. To obtain the maximal possible increase in enzyme activity under the conditions used, we pre-fed the animals with a diet containing 90% glucose and no protein for 4 days.

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EXPERIMENTAL

Male rats of the Sprague-Dawley strain, 6 to 8 weeks old and weighing 150 to 200 g, were fed a protein-free diet consisting of 90% glucose, 5% corn oil, 4% minerals (17) and 1% vitamins (18). Four days later the animals were offered a diet in which casein had been substituted for glucose, but otherwise identical to the previous diet. The rats were killed at zero, 1, 2, 3, 4, and 7 days following the start of the feeding of the high protein diet. The animals were housed individually in screen-bottom cages and were offered food and water ad libitum.

Procedure

Rats were killed by a sharp blow to the head which was followed by decapitation and exsanguination. The livers were removed completely, blotted, weighed, and chilled.

A 20% level homogenate was prepared with ice cold 0.14 M KCl, pH 7.4, using a Potter-Elvehjem homogenizer. Total liver protein was determined in the crude homogenate by a modified biuret procedure (19). The 20% liver homogenate was centrifuged at 20,000 × *g* for 30 minutes and the supernatant solution was used for the determination of soluble protein (19) and all the liver enzyme determinations except glucose 6-phosphatase (9) and phosphorylase (20). The activities of the latter 2 enzymes were determined by measuring the liberation of inorganic phosphate using a 5% whole liver homogenate prepared with cold, 0.1 M potassium citrate buffer, pH 6.5. Liver glycogen was measured by a turbidimetric method (21). The following enzymes were estimated by optical density changes at 340 mμ using the 20,000 × *g* 0.14 M KCl supernatant solution (20); glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, serine dehydrase, fructose 1,6-diphosphatase, L-α-glycerophosphate dehydrogenase, glucose 6-phosphate dehydrogenase, pyruvate kinase and malic enzyme.

The activities of these enzymes were determined by measuring the amount of DPN or TPN reduced or DPNH oxidized. If the enzyme being measured did not require a pyridine nucleotide as an oxidant or reductant, the reaction was coupled to another enzyme or a number of enzymes, one of which did require a pyridine nucleotide as a cofactor for oxidation or reduction. Reagents were used in such concentrations that the activity of an enzyme estimated was linearly proportional to the amount of pyridine nucleotide reduced or oxidized per unit of time. Tyrosine-α-ketoglutaric transaminase activity was estimated by determining the amount of *p*-hydroxyphenylpyruvate formed from tyrosine at 310 mμ (22).

Enzyme activity is reported as units per 100 g body weight. One unit of enzyme was defined as that amount of enzyme which produced one micromole of measured product per minute under the conditions of the enzyme assay.

Phosphorylase and glucose 6-phosphatase assays were conducted at 37°. Other enzyme assays were carried out at 25° using a Gilford Model 2000 multiple sample absorbance recorder.

RESULTS AND DISCUSSION

Rats fed a protein-free diet for 4 days lost an average of 16 g per rat or about 10% of their body weight (table 1). Liver size and liver protein values decreased during the same period to about 60 to 70% of the values obtained with rats of comparable age and sex, but maintained on a 25% casein, 65% glucose diet (12, 14, 20). After being transferred to the high protein diet, the animals started to gain weight rapidly. The high protein diet was readily accepted by the animals and growth was resumed shortly thereafter.

Liver glycogen values of animals receiving the protein-free diet were high. This indicates the animals were accepting the diet. The change to the high protein diet decreased liver glycogen values substantially (table 1).

Under conditions which involve the rapid breakdown of glycogen liver phosphorylase activity would be expected to be high. This was not observed in our experiments, as liver glycogen was low one day after the dietary change, while a noticeable increase in phosphorylase activity occurred much later (table 2). It is possible that a transitory elevation of phosphorylase activity occurred but was not observed, since the breakdown of the stored glycogen could

have been maximal by the time of the first measurement following the change in diet. Also, the possibility exists that the method of killing the animals influenced phosphorylase activity by converting the inactive form of the enzyme into its active form (23), producing an apparently false picture since the active to inactive ratios of the enzyme are of considerable significance in terms of in vivo activity. However, it has been noted by some authors that phosphorylase activity and liver glycogen level are not always correlated (24) and our results tend to support this conclusion.

The activity of both glucose 6-phosphatase (9, 10) and of fructose 1,6-diphosphatase (11) can be increased by a high protein diet. The time course of increase in the activity of these 2 enzymes, however, is different (table 2); glucose 6-phosphatase activity was maximal after 24 hours following the dietary change, while fructose 1,6-diphosphatase activity was still only half-maximal in 2.5 days. This type of regulation of enzyme activity may play an important role in the regulation of blood glucose levels.

The activities of L-α-glycerophosphate dehydrogenase, glucose 6-phosphate dehy-

TABLE 1

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

No. days high protein diet fed	0	1	2	3	4	7
Original body wt, g	167 [1] ± 5 [2]	179 ± 6	182 ± 13	183 ± 9	183 ± 6	172 ± 14
Body wt at time of killing, g	151 ± 5	170 ± 7	174 ± 10	184 ± 9	191 ± 7	197 ± 13
Total liver protein, mg/100 g body wt	1010 ± 27	1160 ± 95	2250 ± 420	2390 ± 510	1990 ± 110	2040 ± 180
Soluble liver protein, mg/100 g body wt	403 ± 15	542 ± 15	737 ± 48	789 ± 72	800 ± 54	891 ± 98
Liver glycogen, mg/100 g body wt	180 ± 26	10.1 ± 4.5	41.1 ± 13	50.0 ± 31	54.0 ± 9.1	83.0 ± 55
Relative liver size (wt liver/body wt at killing) × 100	3.74 ± 0.1	3.62 ± 0.1	4.56 ± 0.1	5.19 ± 0.1	5.30 ± 0.1	5.57 ± 0.4

[1] All values are the average of at least 4 animals.
[2] SE of mean.

TABLE 2

Effect of high protein diet on several liver enzymes of carbohydrate metabolism

No. days high protein diet fed	0	1	2	3	4	7
Phosphorylase	20.0 [1] ± 4 [2]	23.5 ± 6	23.5 ± 4	33.8 ± 4	36.8 ± 14	25.3 ± 9
Glucose 6-phosphatase	67.7 ± 2	109 ± 9	99.0 ± 4	89.6 ± 3	83.9 ± 7	75.6 ± 5
Fructose 1,6-diphosphatase	6.35 ± 0.2	7.90 ± 1	10.3 ± 1	19.0 ± 3	19.9 ± 2	23.1 ± 4
L-α-glycerophosphate dehydrogenase	96.9 ± 8	171 ± 17	193 ± 31	194 ± 16	191 ± 23	217 ± 13
Glucose-6-phosphate dehydrogenase	4.54 ± 0.6	6.13 ± 1	10.0 ± 1	18.0 ± 3	28.2 ± 6	35.9 ± 8
Pyruvate kinase	24.0 ± 2	93.7 ± 16	62.6 ± 19	78.5 ± 8	78.5 ± 17	47.5 ± 9
Malic enzyme	10.6 ± 1	17.8 ± 1	16.7 ± 3	18.4 ± 4	18.1 ± 1	10.8 ± 1

[1] Values are given as micromoles of substrate converted/minute/100 g body weight. All values are the average of at least 4 animals.
[2] SE of mean.

drogenase, malic enzyme and pyruvate kinase increased sharply following the change to the high protein diet (table 2). The activity of L-α-glycerophosphate dehydrogenase did not increase significantly beyond the levels found in animals maintained with a 65% glucose, 25% casein diet (20).

Pyruvate kinase has been studied by Krebs et al. (13), Freedland et al. (14) and others. These authors agree that the activity of this enzyme in the liver is lowered by feeding a high protein, low carbohydrate diet and elevated by a high sugar, low protein diet. Malic enzyme activities can be elevated by feeding a high sugar diet (15). In our experiments the high protein diet increased the activity of pyruvate kinase and malic enzyme compared with the activity of these enzymes in animals fed the high glucose diet (table 2).

It appears likely that other investigators did not observe this type of change in activity, because they include some carbohydrate in their high protein diets (14), or a large amount of fat (13).

It is possible that when the animals were fed the high glucose, protein-free diet, messenger-RNAs specific for the synthesis of pyruvate kinase and malic enzyme were produced at an accelerated rate, but the synthesis of these enzymes was inhibited. This situation could arise either because low concentrations of amino acids in the diet cause a decrease in the synthesis of liver proteins in general, or because messenger-RNA translation preferentially favors the synthesis of other enzymes, or both. Under such circumstance the change to the high protein diet results in an elevation of pyruvate kinase and malic enzyme activity making amino acids available for enzyme synthesis. The elevation in the activity of these enzymes may persist for days, although the production of specific messenger-RNAs is stopped or slowed down by the high protein diet. As the feeding of the high protein diet is continued, the specific messenger-RNAs decay and the activites of pyruvate kinase and malic enzyme decrease.

Another possibility, that the activity of these 2 enzymes increases after the feeding of the high protein diet because of an increase in food intake and an increased internal production of glucose, may not be discounted, though the increase in enzyme activity is accompanied by a reduction in liver glycogen.

Glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, serine dehydrase and tyrosine-α-ketoglutaric transaminase activities can be increased by high dietary protein (4, 6–8, 29). There is good evidence that increased enzyme activity is accompanied by an increased synthesis of new enzyme in the case of tyrosine-α-ketoglutaric transaminase (30, 31) and glutamic-pyruvic transaminase (32).

In our experiments the activities of these 4 enzymes were increased (table 3) from 4.5-fold (glutamic oxalacetic transaminase) to 24-fold (tyrosine-α-ketoglutaric transaminase). Comparison of the percentage of maximal induction versus time after dietary change plots (fig. 1) shows that tyrosine-α-ketoglutaric transaminase is increased faster, whereas the curves are essentially the same for the other 3 enzymes.

TABLE 3

Effect of high protein diet on some liver enzymes of amino acid metabolism

No. days high protein diet fed	0	1	2	3	4	7
Glutamic-oxalacetic transaminase	168 [1] ± 6 [2]	252 ± 12	464 ± 17	696 ± 51	743 ± 29	824 ± 42
Glutamic-pyruvic transaminase	25.3 ± 7	39.0 ± 5	85.3 ± 3	149 ± 10	162 ± 6	206 ± 18
Serine dehydrase	1.74 ± 0.2	4.14 ± 0.8	13.0 ± 2	21.6 ± 1	24.8 ± 4	20.2 ± 4
Tyrosine-α-ketoglutaric transaminase	0.723 ± 0.3	10.5 ± 4	12.3 ± 3	17.0 ± 3	16.7 ± 2	12.3 ± 1

[1] Values are given as micromoles of substrate converted/minute/100 g body weight. All values are the average of at least 4 animals.
[2] SE of mean.

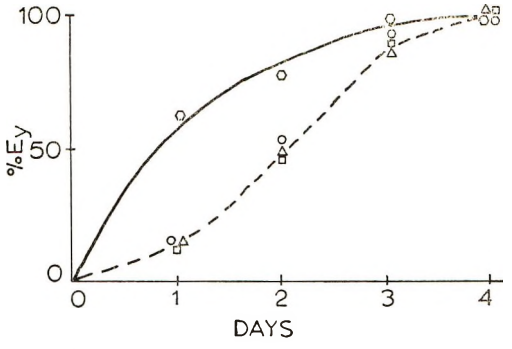

Fig. 1 Time-course of increase of induced activity of rat liver enzymes. % Ey = % induced activity = $100 \times \frac{Ei - Eo}{E_4 - Eo}$. The value of enzyme activity 4 days after the dietary change (E_4) was chosen as 100% induction, the activity before the dietary change (Eo) as 0% induction. *Ei* represents enzyme activity on any given day. Glutamic-oxalacetic transaminase (circle), glutamic-pyruvic transaminase (triangle), serine dehydrase (square) and tyrosine-α-ketoglutaric transaminase (hexagon).

With the possible exception of phosphorylase, all liver enzymes studied increased in activity as early as 24 hours after changing to the high protein diet. The enzymes could be divided into two distinct groups on the basis of the time course of their response to the dietary stimulus. In one group, which contains glucose 6-phosphatase, L-α-glycerophosphate dehydrogenase, pyruvate kinase, malic enzyme and tyrosine-α-ketoglutaric transaminase, enzyme activity reached a maximum within 48 hours after the feeding of the high protein diet (tables 2 and 3). After 48 hours the activity of these enzymes either remained constant or began to decrease with the feeding schedule used. No finer approximation of the time required to reach the maximal activity was attempted. The time course of induction of the other liver enzymes studied appears to be sigmoidal.

Our data provide no positive evidence of increased enzyme activity brought about by the protective action of substrate on enzyme. A definite delay appears to have occurred in the induction of some enzymes. Following this initial delay, the activities of these enzymes increased by a constant amount for 2 days afterwards with the exception of fructose 1,6-diphosphate. It has been suggested by some investigators that a number of enzymes, such as tyrosine-α-ketoglutaric transaminase, have a very rapid turnover rate (29) and therefore an alteration in the rate of enzyme synthesis is noticed immediately in terms of enzyme activity. Other enzymes, such as glutamic-pyruvic transaminase, which has a longer half-life exhibit an apparent delay of induction, because the total amount of enzyme is so large in comparison to the rate of synthesis that an increase in the rate of synthesis may not be detected immediately.[3] A further uncertainty exists as to the mechanism of induction in the case of those enzymes which reached a maximum activity within 48 hours after the dietary change, because the early time course of their induction was not determined.

Further work as to the mechanism of these dietary effects, as well as a more detailed time study, especially in the early course of the increases may provide more information and allow a better understanding of the nature and control of these increases.

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Interrelationships among Copper, Zinc, and Cadmium in the Diet of the Confused Flour Beetle [1,2]

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ABSTRACT Interrelations of dietary copper, zinc, and cadmium have been studied in species such as the mouse, rat, and chicken. It was thought worthwhile, therefore, to run parallel studies with a quite different species, an insect, particularly since most such species do not utilize a respiratory pigment. In the diet of the Confused Flour Beetle, *Tribolium confusum* (Duval), additional copper slightly reduced the toxic effects of high levels of both zinc and cadmium. Although cadmium is highly toxic when only minimal levels of zinc are present, higher levels of zinc are effective in reducing the cadmium toxicity. In terms of requirements of the flour beetle, cadmium in the diet raises the zinc requirement considerably and the copper requirement slightly. A possible competition for enzyme sites is suggested.

There have been numerous reports on the interactions of various minerals. Many are based on their reaction with or similarity to each other. Hill et al. (1), working with chicks, reported that cadmium was toxic at dietary levels of 25 to 400 ppm in a copper and iron-deficient ration. Some of the deleterious effects were overcome by added zinc, others by added copper. Iron also showed marked beneficial effects. A later report [4] from the same laboratory indicated that the addition of zinc, cadmium or silver to a low copper diet produced, by antagonistic action, a presumed copper deficiency syndrome in chicks. They reported further [5] on the effect of copper in overcoming cadmium toxicity. Guggenheim et al.,[6] however, failed to show that an anemia in mice produced with an all-meat diet was due to an unfavorable ratio of zinc and copper.

Several studies have related copper, zinc, and cadmium to iron uptake and storage and to hemoglobin synthesis. Thus Bunn and Matrone (2) showed that addition of 100 ppm of cadmium to diets containing various levels and combinations of copper and zinc reduced liver storage of iron by 30% and hemoglobin by 45%. Growth also was markedly restricted, and it might appear that the marked anemia was the cause of growth failure. It is also possible, however, that the results of these interrelationships might be effected through their effects on metalloenzyme systems, such as cytochrome oxidase, which may contain copper, iron, or zinc as part of the active molecules. It seemed appropriate, therefore, to test the effects of copper, zinc, and cadmium on an organism such as the Confused Flour Beetle which does not utilize respiratory pigments.

METHODS

Newly hatched larvae of *Tribolium confusum* (Duval) were incubated for 18 days as described in previous publications (3, 4). The diet was that used in determining the mineral requirements of this species (4) and had the following percentage composition: cornstarch, 74.0; amino acids, 20.0; corn oil, 3.0; vitamin mixture, 0.63; cholesterol, 0.37; and pure mineral salts, 2.0. The latter mixture was modified to give the desired levels of copper, zinc, and cadmium. This diet had been called the mineral control diet and gave very good growth and reproductive behavior. It supplied 82 ppm of iron from ferrous sulfate

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plus that in the nonmineral ingredients, 1.2 ppm of zinc plus zinc chloride to give 49.2 ppm and 0.12 ppm of copper plus copper sulfate to give 12.8 ppm. Zinc and copper were determined in the basic diet by spectrographic methods. Cadmium levels were calculated based on added cadmium chloride.

RESULTS

Table 1 shows that 2000 ppm of zinc were slightly toxic to growth of flour beetle larvae when a high level (51 ppm) of copper was present, but inhibited larval growth to a much greater extent when very little copper was present in the diet. Thus, a level of copper well above the requirement level but not near the toxic level, which is about 300 ppm, diminished the toxicity due to excess zinc.

TABLE 1

Effect of copper on zinc toxicity, as measured by growth of flour beetle larvae [1,2]

Zn in diet	Larval weight at 18 days	
	Cu in diet	
	51 ppm	0.12 ppm (none added)
ppm	*mg*	*mg*
97	2.64	2.57
481	2.75	2.67
2000	2.03	1.54

[1] Each value represents the average of the average weights of 3 vials containing 10 larvae each. For this number the smallest significant difference at the 1% risk level is 0.21 mg.
[2] Growth with mineral control diet: 2.56 mg.

In table 2, the interrelations of copper and zinc with dietary cadmium are shown. In this experiment, the high levels of copper and zinc are those supplied by the complete mineral control diet while the lower levels are those supplied by the nonmineral constituents. Cadmium was added as cadmium chloride to furnish the indicated levels of the cation. Larvae maintained with a diet containing the higher levels of zinc and copper were not significantly harmed by 150 ppm of cadmium, but growth decreased when the diet contained 400 ppm of cadmium. When the level of copper was reduced to 0.12 ppm, 50 ppm of cadmium was still well-tolerated, but the cadmium became toxic at about the 150-ppm level.

When zinc alone, or both zinc and copper were at low levels (near their minimum requirement levels), as little as 50 ppm of cadmium was not tolerated by the flour beetle larvae. A higher level of copper did little to lessen this toxicity of cadmium in the diet containing a minimum level of zinc. With the higher level of zinc and 150 ppm of cadmium, the high level of copper was also effective. Thus it appears that levels of zinc above the minimum requirement are important in diminishing the toxic effects of added cadmium. Copper may also exert a slight beneficial effect.

Although these data have been discussed in terms of toxicity of the various elements, up to certain levels the effects can also be stated in terms of requirements. Thus it may be said that the addition of cadmium to the diet markedly raises the zinc requirement and raises slightly the copper requirement. The mechanism of action may be competition for enzyme sites as suggested by Vallee.[7] If a given enzyme is saturated by cadmium, then a higher level of zinc, up to a certain point, can

[7] Vallee, B. L. 1963 Metalloenzymes in cellular function. Proc. 6th Int. Congr. Nutr., Edinburgh, p. 58.

TABLE 2

Effects of copper and zinc on cadmium toxicity, as measured by growth of flour beetle larvae [1]

Variable cations		Larval weight at 18 days			
		Level of added cadmium			
Zn	Cu	None	50 ppm	150 ppm	400 ppm
ppm	*ppm*	*mg*	*mg*	*mg*	*mg*
49.2 [2]	12.8 [2]	2.71 [2]	2.63	2.57	1.20
49.2	0.12	2.70	2.63	2.00	
5.0	12.8	2.68	0.95	0.24	
5.0 [3]	0.12 [3]	2.61	0.88	0.15	

[1] Statistical treatment as in table 1.
[2] The mineral control diet.
[3] Levels of Zn and Cu supplied by the diet without added minerals.

overcome the inhibiting effect of the cadmium. Whether any of the changes are manifested through an effect on iron is not known since this element was maintained at a constant level, about 8 times the minimal requirement.

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Effect of Isoniazid on the Urinary Excretion of Taurine by Men

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ABSTRACT Eight young men were maintained with a diet low in vitamin B_6 for 2 months during which the taurine content of the urine was determined. Four of them were administered isoniazid (INH) (mean, 10 mg/kg/day) for the first month. Two of the 4 control subjects excreted less taurine at the end of 2 months than at the beginning. Additional signs of depletion were observed in one of them, subject M. The variability of taurine values was so large that depletion of the vitamin B_6 stores of subject M can only be considered as a possibility. Either three of these subjects had not been given a low vitamin B_6 diet long enough to deplete their stores or a vitamin B_6 deficiency did not cause a decrease in urinary taurine in these subjects. The urinary taurine of the 4 men administered INH increased and remained above the starting values most of the time, even after the termination of INH treatment. The urinary taurine values for 2 of the 4 men responded much more rapidly to INH treatment than those of the other two. The lymphocyte counts in the blood of subject M and of the subjects treated with INH decreased.

The effect of isoniazid (INH) on the metabolic processes of the body is important because many patients with tuberculosis are being treated with the drug. It is known to inhibit the activity of some vitamin B_6-dependent enzymes. The activity of one of them, L-cysteic acid decarboxylase, is known to disappear from the livers of rats in vitamin B_6 deficiency (1) and as a result urinary taurine decreases. If INH simulates a vitamin B_6 deficiency by interfering with the activity of the decarboxylase, presumably a decrease in urinary taurine would result, but contrary to this expectation, an increase in urinary taurine was found by Mercer et al. (2) in a study in our laboratory with rats fed INH. The objectives of the present experiment were to study human subjects: 1) to find whether there is a decrease in urinary taurine excretion when a diet low in vitamin B_6 is fed for 2 months, and 2) to find whether urinary taurine can be used to measure the rate of depletion of vitamin B_6 stores when INH is administered.

MATERIALS AND METHODS

Eight men ranging in age from 22 to 28 years, in height from 171 to 188 cm and in weight from 66.4 to 87.2 kg served as subjects. They were fed a diet of low vitamin B_6 content for 58 to 64 days. The vitamin B_6 content of the diet varied among the subjects from 0.56 to 0.58 mg/day as found by the method of Toepfer and Lehmann (3). In this method the 3 forms of the vitamin are separated by column chromatography and the vitamin B_6 content is determined microbiologically. The calculated nitrogen content of the diet was the same for all subjects. Because they varied in body weight it ranged from 0.17 to 0.24 g/kg body weight.

The INH was administered orally before breakfast, before lunch, and at bedtime to 4 of the subjects for the first 7 4-day periods (28 days). Administration of the drug was started at 2 mg/kg of body weight/day and gradually increased to 15 mg except for subject B. This subject reported itching of the lower extremities and mild nausea when riding in an elevator. These symptoms occurred on days 11, 20 and 25 of administration when the dose of INH had reached 10 mg/kg in each instance. Each time the drug was removed until the reactions disappeared and then started again at the 5 mg/kg level, and the dose gradually increased.

At the conclusion of the study a load dose of 20 mg of pyridoxine hydrochloride

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was administered to all subjects. They continued to eat the low-vitamin B_6 diet for one additional day.

Urine was collected continuously throughout the study and frozen. Taurine was determined either on daily or on 4-day composites by the method of Sörbo (4). The columns of resin used were adequate for the removal of urea: 99.4% of ^{14}C urea, added to human urine, was removed by the columns.

Blood samples were taken immediately before lunch every 2 weeks. Two samples were taken a day apart in the last period, that is, in period 15 for some subjects and in period 16 for the rest. Disodium ethylenediaminetetraacetate (EDTA) was added to prevent coagulation. The pyridoxal phosphate content of leukocytes was determined by 2 methods. While the methods proved to be satisfactory for use with rat blood, the normal values for human blood are so low that the methods were not sufficiently sensitive; consequently, the results are not reported. Differential counts were made on 2 slides counting 200 leukocytes on each. From this and the total number of leukocytes per cubic millimeter the number of lymphocytes per cubic millimeter was calculated.

RESULTS

In the first 4-day period the taurine content of the urine of the 8 men ranged from 91 to 188 mg/24 hours. The excretion during the first period was taken as the basal level and the amount of taurine excreted in later periods was compared with the initial value for each subject (figs. 1 and 2).

Effect of a low vitamin B_6 diet on urinary taurine. Two of the four subjects, F and cM, who ate the vitamin B_6-low diet, and were not administered INH, exhibited little evidence of a consistent decrease in taurine excretion over the 15 periods (57 days for subject cM) (fig. 1).

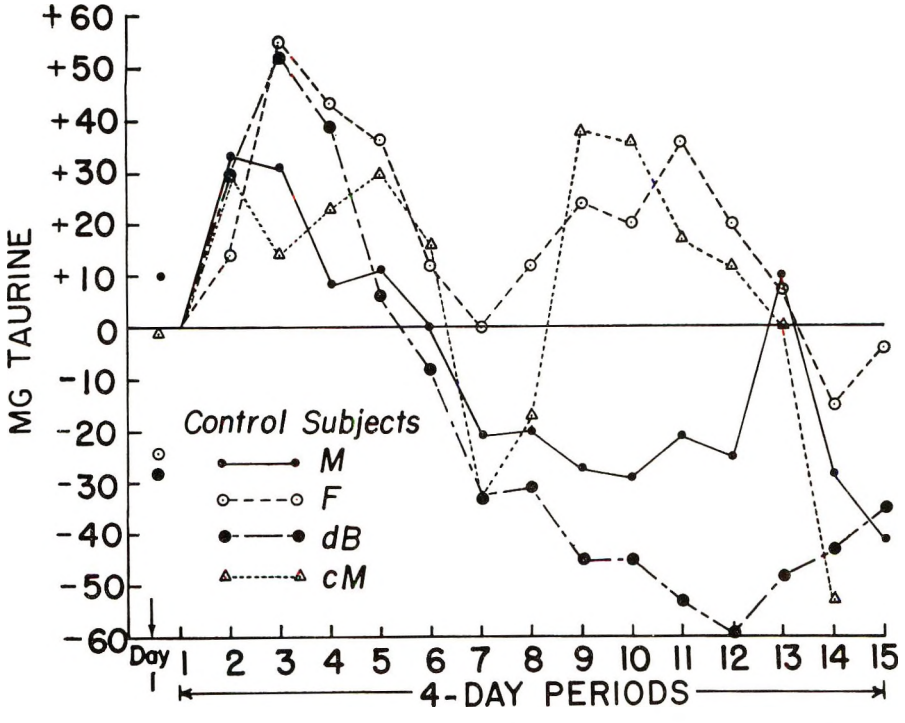

Fig. 1 The deviation of values for the mean daily urinary taurine in each 4-day period from the mean value for the first period for 4 men given a diet low in vitamin B_6 (control subjects). Also, the deviation of the value for the first day from the mean for period 1.

After a brief increase at first the amount of taurine in the urine of the other 2 subjects (M and dB) decreased. The values for one of them, subject M, decreased from 147 to 106 mg/24 hours (fig. 1), a decrease which may be meaningful. Other indexes supported the hypothesis that his body stores of vitamin B_6 were being depleted. Following the load dose of pyridoxine hydrochloride, the taurine content of his urine rose to 176 mg/24 hours from 106 mg in period 15. The oxalic acid content of his urine increased from 31.7 in period 1 to 39.0 mg/24 hours in period 15, an increase of 23%. A regression of oxalic acid with time was highly significant ($P < 0.01$). His lymphocyte count decreased from 3,460 cells/mm³ (table 1) in the week before the study began to a mean of 2,540 cells/mm³ for 2 succeeding days in period 16. Near the end of the study he became nauseated when blood samples were taken. Before the study he had been eating a diet containing less vitamin B_6 than the other subjects.

Taurine values for subject dB decreased even more than those for subject M beginning with period 5 but rose in the last 3 periods (fig. 1). None of the changes in other indexes noted with subject M was large enough to indicate depletion of vitamin B_6.

Effect of INH on urinary taurine. Contrary to what would be expected if INH interferes with or reduces the pyridoxal phosphate needed by the enzyme which

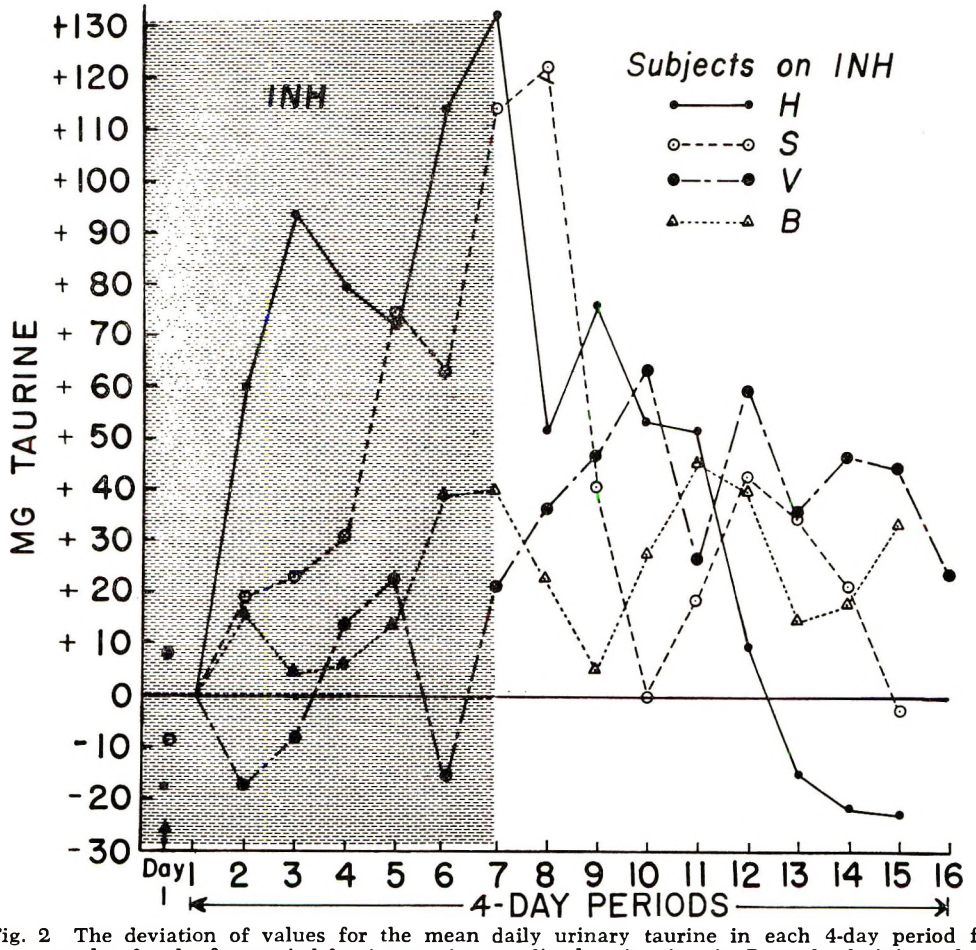

Fig. 2 The deviation of values for the mean daily urinary taurine in each 4-day period from the mean value for the first period for 4 men given a diet low in vitamin B_6 and administered INH for the first 7 periods. Also, the deviation of the value for the first day from the mean for period 1.

decarboxylates L-cysteic acid, INH caused an increase of taurine in the urine of the 4 subjects who were administered the drug. Inspection of figure 2 shows that most of the time during INH treatment and afterwards the 4 subjects were excreting more taurine than in period 1.

Mean values for the 4 subjects increased during INH treatment and a regression with time was highly significant ($P < 0.01$). After termination of INH treatment mean values decreased progressively and a regression with time was highly significant ($P < 0.01$). The increase and later decrease were caused mainly by subjects H and S.

Subjects H and S responded more rapidly to INH administration than the other 2 subjects. During the 7 periods (28 days) of INH treatment, values for subject H rose from 108 to 240 mg/24 hours or 128% and values for subject S rose from 91 to 205 mg/24 hours or 125%. When INH treatment was stopped the taurine content of the urine dropped precipitously (fig. 2). Apparently an effect of INH continued after administration ended because values for subject H remained above those for the first period until period 13. Even after that the urinary taurine excretion was essentially the same as for the first day (fig. 2) of the first period. During the first 4 days values increased so rapidly that the mean was raised by INH. After subject S terminated INH treatment, only 2 values out of 8 (fig. 2) were below those for the first period.

The other 2 subjects (V and B) did not respond as rapidly. They also evidenced a delayed effect of INH because all of their values remained above the base period after treatment with INH was terminated. The difference in response of subjects V and B from that of subjects H and S to the administration of INH is not unexpected because people are known to react differently to the drug. Evans et al. (13) and Dufour et al. (14) report that people can be divided into generic groups depending upon the rate at which they inactivate the drug.

One of the objectives of this study was to determine whether urinary taurine can be used as a criterion of vitamin B_6 depletion during INH treatment. Obviously it cannot.

Urinary taurine excretion after a load dose of pyridoxine hydrochloride. If pyridoxal phosphate-requiring enzymes are needed for the main path of metabolism in which taurine is formed, a load dose of pyridoxine hydrochloride would be expected to cause an increase in urinary taurine in cases where the supply of pyridoxal phosphate in the body had been depleted. A large increase of taurine (70 mg/24 hours) occurred in the urine of subject M following a load dose of 20 mg of pyridoxine hydrochloride. The urinary taurine of subject F increased by 39 mg; for 3 subjects increases were small (24, 14, 9 mg), whereas for 3 others the values decreased slightly (3, 5, 10 mg). The large increase in the urinary taurine of subject M may be an indication of reduced stores of vitamin B_6.

Effect of a low vitamin B_6 diet and of INH on lymphocyte count. Of the 4 control subjects the lymphocyte count of only subject M declined from the week before he started the control low vitamin B_6 diet to the end of period 16: a decrease from 3,460 (table 1) to 2,540 cells/mm^3 (mean for counts made on 2 successive days). Counts for subjects F and cM were about the same at the end as at the beginning (table 1). The leukocyte and lymphocyte counts made on the blood of subject dB were high, so high as to be outside the normal range, but the medical advisor could find no cause for it; the subject's values fluctuated in an unaccountable way. The mean of counts on the blood of 4 subjects, one of whose counts fluctuated irrationally, and one whose counts decreased much, is of little value in interpreting the results. Cheslock and McCully (16) found a decrease in lymphocytes in the blood of 5 of 8 subjects after receiving a low vitamin B_6 diet for 7 weeks. Lymphocytopenia was noted by Vilter et al. (17) who induced a vitamin B_6 deficiency in human subjects with deoxypyridoxine.

A decided depression in the lymphocyte counts in the blood of 3 of the 4 subjects treated with INH occurred after INH had been administered for 2 weeks (in the fourth 4-day period). Mean counts decreased from 3,460 to 2,260 cells/mm^3

TABLE 1

Effect of a low vitamin B_6 diet and of isoniazid (INH) on lymphocyte count

Subject	Week before dietary period	Lymphocyte counts of whole blood						
		4-day diet periods in which blood was drawn						
		4		7		11	15 and 16 [1]	
		Without INH	With INH	Without INH	With INH	Without INH	Without INH	Without INH
		cells/mm³ whole blood						
Control:								
F	2,820	3,210		2,390		2,160	2,780	2,880
M	3,460	3,570		2,520		2,030	2,620	2,470
dB [2]	4,890	6,580		6,530		6,780	4,640	4,690
cM	2,700	2,580		2,150		2,100	2,720	2,620
Mean	3,470	3,980		3,400		3,270	3,190	3,160
Treated:								
B	3,980		2,450		2,970	2,680	2,810	2,610
V	3,340		1,970		3,070	3,450	2,550	2,340
H	3,770		1,840		2,320	2,040	2,530	2,360
S	2,740		2,800		2,740	5,310 [3]	3,040	2,810
Mean	3,460		2,260		2,780	3,370	2,730	2,530

[1] Blood for these counts was drawn one day apart for each subject.
[2] Subject dB had a high leukocyte and lymphocyte count in his blood throughout the study. The medical advisor could find no cause for this.
[3] The leukocyte count in the blood of subject S was 11,810 cells/mm³. It was probably high because he ran to be on time to have his blood sample taken.

(table 1). On the last day of the seventh 4-day period the counts for the 3 subjects whose lymphocyte counts had originally decreased improved (table 1) but the increase was not enough to restore counts to the level of the pretreatment week. The mean for 2 successive days in periods 15 and 16 was 2,630 cells/mm³ while the mean count for the pretreatment week was 3,460 cells (table 1) or a decrease of 830 cells. Possibly INH or INH plus a low vitamin B_6 diet decreased the number of lymphocytes in the blood but such a conclusion should be drawn with caution as the final mean count of 2,630 cells/mm³ is not considered to be low. One might speculate that for some reason some of the counts in the preliminary week were high rather than that those at the end were low. Stress, certain infections, and physical exercise are known to raise lymphocyte counts. The temporary decrease after 2 weeks of INH treatment was more convincing than the decrease at the end of the study. No reports were found in the literature regarding the effect of INH on lymphocytes.

DISCUSSION

Three studies regarding the urinary excretion of taurine by human subjects eating low vitamin B_6 diets were found in the literature. Scriver and Hutchinson (5) observed an infant with convulsions which could be controlled by vitamin B_6 therapy. Following the administration of the vitamin, urinary taurine increased. In a study by Merrow et al. (6) with 8 men and one by Swan et al. (7) with 6 men, all living on controlled diets containing little vitamin B_6, urinary taurine did not decrease significantly after 48 days in the former and 25 or 35 days in the latter. From these 2 studies and the present one, the conclusion is either that all the subjects except possibly subject M had not become sufficiently depleted of vitamin B_6 to cause a decrease in urinary taurine or that human beings do not need vitamin B_6 to form taurine as do rats. Jacobsen and Smith (8) reported that the human liver, unlike the liver of the rat, lacks any appreciable amount of either cysteic acid decarboxylase or cysteine sulfinic acid decarboxylase which require pyridoxal phosphate as a coenzyme. L-Cysteic acid and L-cysteine sulfinic acid are the recognized precursors of taurine in some species of animals. Two investigators (9, 10) have produced partial evidence that taurine may be formed by pathways which do not require pyridoxal phosphate.

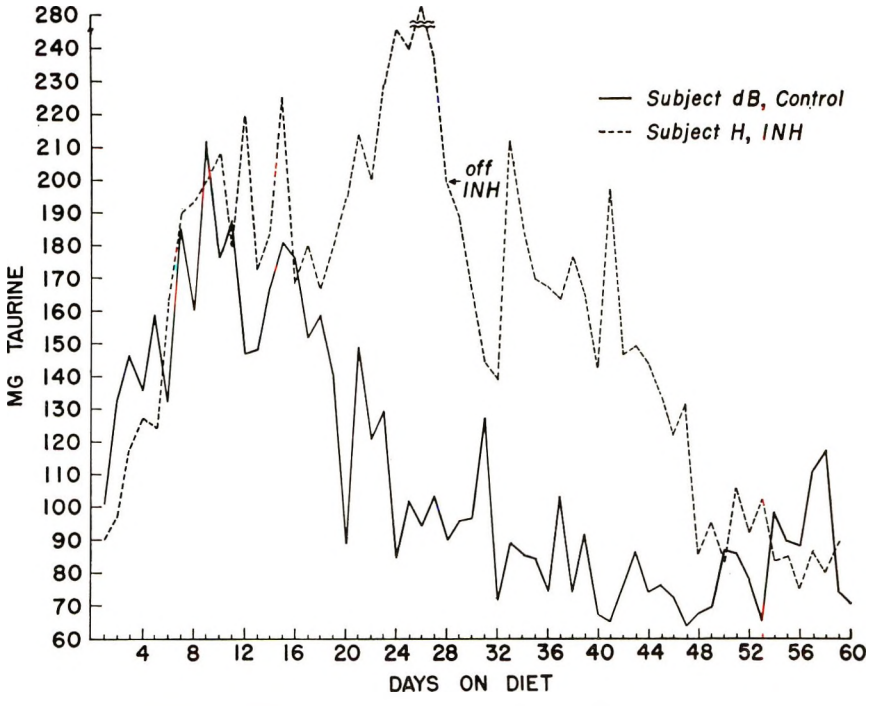

Fig. 3 Daily urinary taurine values for 2 subjects.

Great fluctuation of taurine from one 4-day period to another will be noted in figures 1 and 2: still greater fluctuation is shown in figure 3 in which daily taurine values for subjects dB and H have been plotted. The reason for this variability is not known: one possibility is stress. Brooksby [1] found an increase in urinary taurine of 2 men confined in a simulated space cabin for 7 days. Levenson et al. (11) found high urinary taurine in patients with severe battle wounds and others have found large increases in taurine excretion caused by major operations. Cortisone is known to increase urinary taurine. The increase in the taurine content of the urine of all 4 control subjects in period 2 or periods 2 and 3 (fig. 1) may have been caused by the stress of adjusting to the conditions of the metabolic study; the increase in the taurine values of the subjects treated with INH (fig. 2) may have been caused by both stress and INH.

Another cause of the increase of taurine in periods 2 and 3 may have been the adjustment to a diet of higher sulfur-amino acid content than that of the customary diets of the subjects. In addition to 2.7 g/day of sulfur-amino acids in the food in the experimental diet as found by calculation, there were 2 g of DL-methionine and 1 g of L-cystine in an amino acid supplement. The average per capita consumption of sulfur-amino acids in the U.S.A. is 3.4 g/day (12).

The way in which INH acts to increase urinary taurine is not definitely known. If human beings react to INH as do rats, a possible cause is an increase of pyridoxal phosphate in the liver. An increase in the pyridoxal phosphate content of the livers of rats fed INH in our laboratory has been reported by Sevigny et al. [(15) exp. B and C]. The possibility of hydrazone formation with pyridoxal phosphate and whether a hydrazone would prevent the activity of the coenzyme has been discussed by Mercer et al. (2). They suggested other possible causes for an increase of taurine excretion caused by INH such as: a toxic effect not related to the anti-

[1] Personal communication, G. A. Brooksby, Life Support Branch, Ames Research Center, Moffet Field, California.

vitamin effect, an increase in the excretion of preformed tissue taurine, and action of the adrenal cortex.

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Some Effects of Amino Acids and Cortisol on Tyrosine Toxicity in the Rat [1]

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ABSTRACT An excessive tyrosine intake causes growth depression and paw and eye lesions in young rats fed a low protein diet. The objective of this investigation was to determine the effects of various amino acid supplements and of cortisol injections on the development of tyrosine toxicity syndrome in rats fed a high tyrosine diet. Plasma tyrosine concentration, liver tyrosine transaminase activity and excretion of tyrosine metabolites were measured in rats receiving the amino acid supplements. Supplements of glycine, methionine, tryptophan or a mixture of leucine, isoleucine and valine as well as injections of cortisol and Celite prevented the developmnt of the pathological lesions. Threonine supplementation prevented the growth-depressing effect of high dietary tyrosine and also the development of lesions. High tyrosine diets increased adrenal weight. Cortisol and Celite injections as well as various dietary supplements of amino acids decreased plasma tyrosine concentration. A high tyrosine intake increased liver tyrosine transaminase activity and some amino acid supplements caused further increases in the activity of the enzyme. However, the changes in tyrosine transaminase were not consistent and did not appear to be related to changes in plasma tyrosine concentration. A high tyrosine intake also resulted in increased total phenol excretion. The various amino acid supplements did not increase it further. After 3 weeks of feeding threonine and glycine supplements there was a substantial increase in homogentisic acid excretion in urine which suggests that the rate of catabolism of tyrosine is increased by such treatments.

When rats are fed a low protein diet containing an excessive amount of tyrosine their growth is retarded and they develop lesions of the paws and eyes (1–4). Many attempts have been made to alleviate tyrosine toxicity. Sullivan et al. (1) reported that cystine reduced the severity of the growth depression and pathologic lesions in rats fed a high tyrosine-low protein diet. Glycine improved the condition of animals fed diets containing 10% tyrosine; tryptophan also had some beneficial effect (3). Addition of 1.25% of L-threonine to a diet containing 3% of L-tyrosine alleviated both growth depression and pathologic lesions (5).

The way in which tyrosine toxicity is alleviated by these compounds is not known. Supplementation of the high tyrosine-low protein diet (5) with threonine caused a substantial decrease in plasma tyrosine concentration (6) and hence the question arises whether the various agents that exert some protective effect also lower the concentration of tyrosine in body fluids either through enhancing the excretion of tyrosine and its metabolites or through stimulation of oxidation of tyrosine. Since tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, E.C. no. 2.6.1.5), the enzyme catalyzing the initial step in tyrosine degradation, is elevated in rats subjected to various dietary and hormonal treatments, the possibility exists of a relationship between the activity of this enzyme and the occurrence of tyrosine toxicity in rats fed a high tyrosine diet.

Hydrocortisone injection can result in more than a fourfold increase in tyrosine transaminase activity within a short period of time (7, 8). Other treatments, such as Celite injection, that initiate stress reactions, also cause elevations in the activity of this enzyme (8). Munro and associates (9, 10) have reported that injection or feeding of certain amino acids stimulates

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corticosterone production. If the amino acids that alleviate tyrosine toxicity also stimulated corticosterone production and, thereby, tyrosine transaminase activity, this might enhance tyrosine catabolism and thereby lower plasma tyrosine concentration.

As part of a study of the basis for the alleviation of tyrosine toxicity, we have examined the effects of several amino acid supplements and of injections of cortisol on growth, adrenal weight and on the signs of toxicity of rats fed a high tyrosine diet. Plasma tyrosine concentration and liver tyrosine transaminase activity were estimated at different times during the feeding trials with the amino acid supplements; also, total phenol and homogentisic acid excretion were estimated.

EXPERIMENTAL

Animals. Young male Holtzman or Sprague-Dawley strain rats, weighing 55 to 65 g, were used. Animals were housed individually in suspended cages. Food and water were given ad libitum.

Diets. All the diets were fed as agar-gels containing about 50% of moisture as described previously (6). The composition of the basal diet was: (in % dry weight) casein, 6.0; L-methionine, 0.2; salt mixture, 4.0 (11); vitamin mixture, 0.5 (12); choline chloride, 0.2; corn oil, 5.0; agar, 2.5; dextrin, 40.8; and sucrose, 40.8. Other amino acids were added at the expense of carbohydrate. The diets were stored at 4° throughout the experiments.

Injections and rating of symptoms. Celite [4] and cortisol [5] were suspended in physiological saline. Intraperitoneal injections of 0.3-ml volume containing the amounts indicated in the tables were given daily between 2 to 5 PM.

The procedure for rating the toxicity symptoms was that reported previously (5), with zero indicating no lesions and 10 maximal severity.

Plasma tyrosine determination. Rats were anesthetized with ether and blood was drawn by heart puncture. In the experiments on the effects of different amino acids on tyrosine toxicity, plasma tyrosine concentration was determined after 2, 10 and 21 days of feeding. The method used was that adapted by Udenfriend and Cooper (13).

In the experiments on the effects of cortisol on tyrosine toxicity, plasma tyrosine concentration was determined together with other amino acids using a Technicon automatic amino acid analyzer.

Assay of liver tyrosine transaminase. Livers were removed and frozen at − 20°. On the day of the assay, liver samples were homogenized in 0.14 M potassium chloride containing 0.005 N sodium hydroxide. The enzyme activity was determined using the method described by Rosen et al. (14) which is a modification of the method of Canellakis and Cohen (15).

Urine analyses. Groups of 4 rats each were kept in stainless steel metabolic cages. Urine was collected under hydrochloric acid over 2-day periods, that is, days 6 and 7, 12 and 13, and 21 and 22 of the experimental period. Total phenols in the hydrolyzed urine were determined by the method used by Bernhart and Zillikin (16). Homogentisic acid was determined in the unhydrolyzed urine using the iodine microtitration of Neuberger (17). Values are expressed per 100 g body weight.

RESULTS

Effect of cortisol and Celite on tyrosine toxicity. The effects of daily injections of cortisol or Celite on the growth of rats fed the high tyrosine diet are shown in figure 1. Although 1 mg cortisol per day had no effect on the weight gain of the control group, it improved that of the group fed the high tyrosine diet. However, injections of 6 mg per day of Celite or 5 mg per day of cortisol did not improve the growth of rats fed the high tyrosine diet; but, both of these treatments prevented the development of pathologic lesions (table 1). The higher amount of cortisol will cause severe growth depression of rats fed the control diet, and hence its failure to improve growth of those fed the high tyrosine diet is not unexpected.

Ingestion of the high tyrosine diet with or without Celite injection increased adre-

[4] Celite 545, Johns-Manville Products Corporation, Minneapolis.

[5] Hydrocortisone acetate, Mahady Company, Cambridge, Massachusetts.

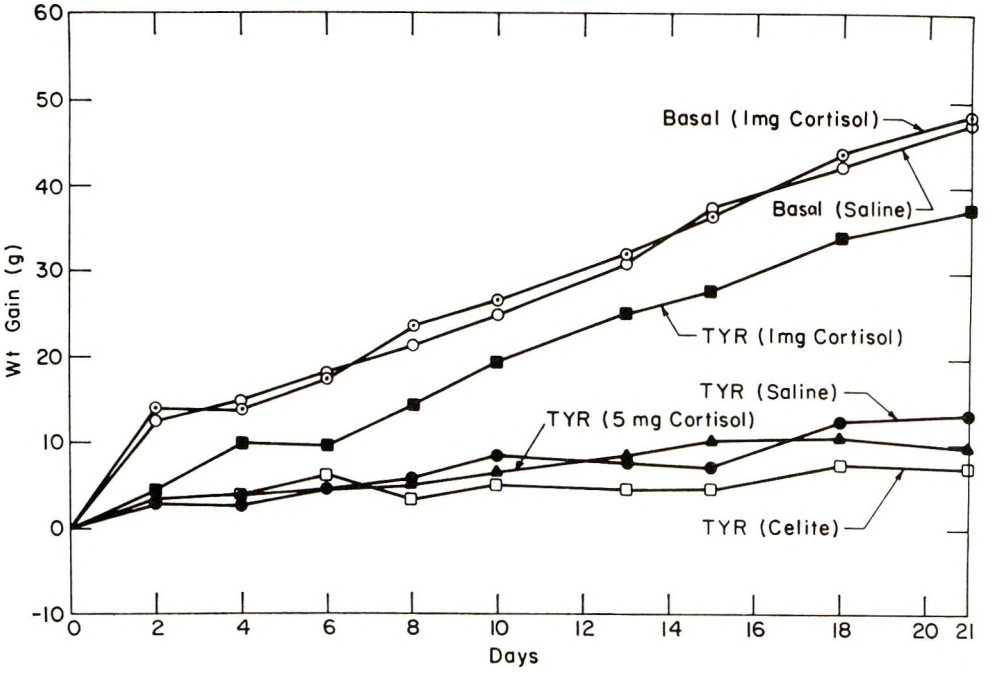

Fig. 1 Effect of daily intraperitoneal injections of cortisol acetate or Celite on the growth of rats fed a diet containing 6% of casein and 3% of L-tyrosine.

TABLE 1

Effects of cortisol and Celite on the severity of eye and paw lesions of rats fed 3% L-tyrosine and on adrenal weight after 24 days [1]

Additions to 6% casein basal diet	Treatment	Eye and paw lesions			Adrenal wt	Plasma tyrosine [2]
		Incidence	Severity			
			Eye	Paw		
					mg/100 g body wt	*μmole/100 ml*
None	saline	0	0	0	17±1(5) [3]	5
None	cortisol (1 mg)	0	0	0	15(2)	6
3% L-tyrosine	saline	5	2.0	7.0	29±2(4)	302
3% L-tyrosine	cortisol (1 mg)	3	3.7	2.6	18±3(3)	283
3% L-tyrosine	cortisol (5 mg)	0	0	0	17±2(4)	63
3% L-tyrosine	Celite (6 mg)	0	0	0	33±1(4)	34

[1] Five rats/group from Holtzman Company with initial wt 75 to 80 g were used. Cortisol and Celite were injected intraperitoneally in 0.3 ml saline each day, the control rats were injected with 0.3 ml saline/day.
[2] Plasma of 5 rats was pooled.
[3] SE of mean. Number of observations in parentheses.

nal weight (table 1); cortisol injection prevented the increase in adrenal weight.

Daily injections of Celite or 5 mg of cortisol greatly reduced the concentration of plasma tyrosine in rats fed the high tyrosine diet. However, daily injections of 1 mg of cortisol did not significantly decrease plasma tyrosine concentration.

Effects of amino acid supplements on growth and food intake. All rats consuming the high tyrosine diet grew slowly (table 2). Within one week they had all developed signs typical of tyrosine toxicity, including edema and reddening of the paws and encrustations of a dark exudate around the eyes. Threonine was the only amino acid that alleviated both the growth depression and the external lesions. The supplement of 1.25% of L-threonine also increased markedly the food intake of rats

fed the high tyrosine diet. Of the rats receiving the other amino acid supplements only one, in the methionine-supplemented group, exhibited any signs of toxicity. Weight gain and food intake were not appreciably improved by any of these supplements (table 2).

Adrenal weight and amino acid supplements. The adrenal weights recorded after 3 weeks are also shown in table 2. Adrenals damaged during removal were not included in calculating the averages. Adrenals of rats fed the diet containing 3% of L-tyrosine were much larger than those of the controls. Threonine was the only amino acid that substantially decreased the adrenal weight of rats receiving 3% of L-tyrosine.

Effects of different amino acid supplements on plasma tyrosine concentration and liver tyrosine transaminase of rats fed high tyrosine diet. Plasma tyrosine concentration of rats fed the diet containing 3% of L-tyrosine was greatly elevated at 2 days, 10 days and 3 weeks (table 3). The addition of the various amino acids to the high tyrosine diet resulted in a lowering of plasma tyrosine concentration and this effect persisted throughout the experiment. It seems unlikely that this was due to low tyrosine intake as food intakes of all groups except for that receiving tyrosine and glycine were as high as or higher than that of the high tyrosine group.

Within 2 days there was a marked increase in liver tyrosine transaminase activ-

TABLE 2

Effect of dietary additions of several amino acids on weight-gain and food intake of rats fed excess of tyrosine

Additions to 6% casein basal diet		Avg wt gain	Avg food intake	Avg adrenal wt
L-Tyr	Other amino acids			
%		*g/3 weeks*	*g/day*	*mg/100 g body wt*
—	—	47±2 [1]	8.6	16±1
3.0	—	12±1	5.3	31±2
3.0	1.25% L-Thr	38±2	7.0	21±1
—	2.5% Gly	30±4	6.2	20±2
3.0	2.5% Gly	12±2	4.6	29±1
3.0	1.0% L-Met	18±3	6.0	32±2
3.0	5.0% L-Leu + 0.2% L-Ile + 0.2% L-Val	16±2	5.1	30±1
3.0	1.0% L-Trp	14±2	5.0	29±2

[1] SE of mean of 4 rats/group.

TABLE 3

Effect of different amino acids on liver tyrosine transaminase and plasma tyrosine concentration of rats fed 3% L-tyrosine

Diet	2 days		10 days		21 days	
	Plasma Tyr [1]	Enzyme activity [2]	Plasma Tyr [1]	Enzyme activity [2]	Plasma Tyr [1]	Enzyme activity [2]
	μmole/100 ml plasma		*μmole/100 ml plasma*		*μmole/100 ml plasma*	
6% casein+0.2% L-Met(1)	2.5	44.0± 2.7 [3]	4.2	44.3± 2.4	4.6	47.2± 6.0
(1)+3.0% L-Tyr(2)	359.0	84.4±10.8 [4]	471.0	135.6±29.3 [4]	164.8	78.8± 8.8 [4]
(2)+1.25% L-Thr	200.0	90.6±28.8	130.0	56.9±28.2	31.0	79.9±22.7
(1)+2.5% Gly	3.3	122.8±24.0 [4]	21.0	94.6±15.3 [4]	1.8	90.9± 5.1 [4]
(2)+2.5% Gly	76.5	129.9± 5.3 [4]	73.7	125.3±12.7 [4]	26.3	153.4±19.1 [4]
(2)+1% L-Met	154.0	100.4±24.7	291.0	95.6±32.5	102.6	120.5±25.2 [4]
(2)+5.0% L-Leu+ 0.2% L-Ile+0.2% L-Val	26.0	82.7± 9.3 [4]	90.1	137.5±24.1 [4]	61.9	103.5±17.6 [4]
(2)+1.0% DL-Trp	72.0	121.4±22.4 [4]	65.8	86.8±17.0	70.0	150.5±39.8

[1] Average of 3 to 4 rats/group.
[2] Micromoles of *p*-hydroxyphenylpyruvic acid formed/g liver/hour.
[3] SE of mean.
[4] Significantly different from the control at a 5% level.

ity of rats receiving tyrosine alone or with the various amino acid supplements (table 3). However, the degree of increase varied from period to period. The enzyme activity of the high tyrosine group rose then fell at the end of 3 weeks, whereas the values for the tyrosine-glycine and tyrosine-tryptophan groups were highest at the end of the 3-week feeding period. At 21 days the values for groups receiving the various supplements other than threonine tended to be above that for the high tyrosine group.

Effect of different amino acids on total urinary phenol excretion. During the first 2-day collection period the control group excreted 11 mg of phenols per 100 g body weight. Feeding excess tyrosine increased the excretion of total phenols in urine (table 4) with the value for the methionine-supplemented group being highest. During the second period total phenol excretion by rats fed tyrosine with or without methionine increased substantially. However, urinary total phenol excretion by animals fed 1.25% L-threonine or 2.5% glycine did not increase; in fact, excretion by the threonine-supplemented group which had the highest tyrosine intake actually decreased.

By the end of the experimental period, there was a decrease in the total phenol excretion of rats fed the 3% L-tyrosine diet but the amount was still higher than that of any of the other groups. The methionine-supplemented group showed a marked decrease in total phenol excretion during the last period.

Effect of different amino acids on urinary homogentisic acid excretion. Feeding tyrosine alone or together with the other amino acids for one week did not cause a rise in urinary homogentisic acid excretion above the value for the control group (table 5). There was a slight increase in urinary excretion in rats fed the glycine supplement during the 12- to 13-day period. However, by the end of the experimental period, that is, after 3 weeks of feeding the experimental diets, rats receiving threonine or glycine supplements showed a substantial increase in homogentisic acid excretion.

TABLE 4

Effect of different amino acid supplements on body weight,[1] tyrosine intake,[2] and total phenol excretion[3] of rats fed 3% L-tyrosine[4]

Diet	Days 6 and 7			Days 12 and 13			Days 21 and 22		
	Avg wt of rats	Tyr intake	Total phenols	Avg wt of rats	Tyr intake	Total phenols	Avg wt of rats	Tyr intake	Total phenols
6% Casein + (0.2% L-Met)(1)	70.1	50	11	82.9	59	7	95.8	48	8
(1) + 3% L-Tyr	63.6	555	79	66.5	436	221	72.1	517	154
(1) + 3% L-Tyr + 1.25% L-Thr	70.1	636	73	83.4	530	37	100.0	508	51
(1) + 3% L-Tyr + 1% L-Met	62.3	650	157	72.0	528	243	77.0	505	69
(1) + 3% L-Tyr + 2.5% Gly	65.1	502	42	70.7	481	55	76.0	568	63

[1] Average grams of body weight of 4 rats during the 2-day collection period.
[2] Milligrams of tyrosine consumed/100 g body weight in the 2-day collection period.
[3] Milligrams equivalent to *p*-hydroxyphenylpyruvic acid excreted in urine/100 g body weight.
[4] Average body weight of 4 rats at start being 60.6 g. Seven milligrams of total phenols were excreted by control rat during days 1 and 2.

TABLE 5

Effect of different amino acids on urinary homogentisic acid excretion[1] of rats fed 3% L-tyrosine

Diet	Days 6 and 7	Days 12 and 13	Days 21 and 22
6% casein (+0.2% L-Met)(1)	2.4	2.1	2.0
(1) + 3% L-Tyr	2.4	3.2	2.9
(1) + 3% L-Tyr + 1.25% L-Thr	2.4	5.0	28.8
(1) + 3% L-Tyr + 1% L-Met	2.6	3.5	5.7
(1) + 3% L-Tyr + 2.5% Gly	3.2	7.1	22.9

[1] Milligrams/100 g body weight excreted during the 2-day collection period.

DISCUSSION

Tyrosine transaminase, the catalyst for the first reaction on the main pathway of tyrosine catabolism, is elevated in rats injected with cortisol or Celite (7, 8); such a response might be expected to enhance tyrosine catabolism. This would be consistent with the present results as plasma tyrosine concentration was only moderately elevated and pathologic lesions did not develop in rats fed the high tyrosine diet and injected daily with 5 mg of cortisol or 6 mg of Celite. Celite presumably increases the blood level of corticosterone by stimulating endogenous production. Injection of 1 mg of cortisol did not prevent the lesions caused by 3% of tyrosine. This is of interest in relation to the observations of Rivlin (18) who found that a dose of 1 mg of cortisol did not induce liver tyrosine transaminase in hyperthyroid rats although a 5 mg dose did. Since tyrosine is a thyroxine precursor the possibility that a high tyrosine diet stimulates thyroxine production should be examined.

Munro and associates (9, 10) have reported that plasma corticosterone increases if rats are fed a meal containing 1 g of either methionine or leucine and that prolonged feeding of these 2 amino acids increases adrenal corticosterone concentration. Neither Celite nor the amino acid supplements increased adrenal weight above the value for the high tyrosine group, yet, like 5 mg of cortisol, all these supplements prevented the rise in plasma tyrosine concentration and the development of pathologic lesions. It is possible that these supplements act as an additional stimulus and enhance corticosterone production or release even though they do not cause further enlargement of the adrenal glands.

The activity of tyrosine transaminase was elevated in all groups fed high tyrosine diets, as would be expected from the fact that the activity of this enzyme increases in rats injected with cortisol or fed large amounts of tyrosine (7, 8, 19). However, there is obviously no direct relationship between plasma tyrosine concentration and the activity of the transaminase, even though enzyme values for groups receiving amino acid supplements other than threonine tend to be above that for the high tyrosine group at 2 and at 21 days. Knox et al. (20) have reported that the severity of tyrosyluria in rats fed a high tyrosine diet depends not only upon the activity of tyrosine transaminase but also upon the relative activities of tyrosine transaminase and *p*-hydroxyphenylpyruvate oxidase.

All of the dietary additions of amino acids that prevented the development of pathologic lesions in rats fed these high tyrosine diets also prevented the usual rise in plasma tyrosine concentration. In fact, there is a close relationship between plasma tyrosine concentration and the occurrence of signs of tyrosine toxicity. High plasma tyrosine was associated with the occurrence of severe signs in rats fed the 3% tyrosine diet; 1 mg of cortisol did not alleviate the signs nor lower plasma tyrosine concentration; 1% of L-methionine which was the least effective amino acid supplement caused the least lowering of plasma tyrosine concentration; all the treatments that prevented the appearance of toxicity signs lowered plasma tyrosine concentration markedly. Also, plasma tyrosine decreased substantially by day 21 in the group fed the high tyrosine diet and this coincides well with the time of regression of the lesions in rats fed this diet. There was no obvious relationship between tyrosine intake and plasma tyrosine concentration as several of the groups with low plasma tyrosine concentrations consumed more tyrosine than the high tyrosine control group.

The development of alcaptonuria, that is, excretion of homogentisic acid in rats fed the threonine and glycine supplements is of interest. As a matter of fact, the amounts of homogentisic acid excreted during the last period represent a significant amount of the total phenols excreted during this period by such animals. Increased homogentisic acid excretion may indicate that the previous enzymatic steps are rapid and that further oxidation of homogentisic acid then becomes limiting. This view is favored by the previous finding that the addition of glycine to the 3% L-tyrosine diet has resulted in a substantial increase in tyrosine-α-ketoglutarate transaminase activity which was accompanied by a substantial decrease in plasma tyrosine concentration. In this connection the effects of glycine on the enzyme activity and the plasma tyrosine concentration were more

pronounced than those of threonine supplementation.

Overall, the results indicate that supplements of certain amino acids alleviate the external lesions that develop in rats fed a low protein diet containing 3% of L-tyrosine and that this beneficial effect is not mediated through increased urinary excretion of tyrosine and its metabolites. Injections of cortisol and Celite can similarly alleviate the lesions but whether the beneficial effects of the amino acid supplements are directly related to enhanced corticosterone production must await further investigation.

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Absorption and Metabolism of Dietary Triglycerides in Germfree and Conventional Rats [1]

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ABSTRACT Although it is well-known that the physiology, morphology and biochemistry of the germfree animal differs in many respects from its conventionally reared counterpart, these studies were initiated to investigate the effect of the absence of the intestinal microflora on the absorption and metabolism of triglycerides derived from dietary corn oil. Germfree and conventional male rats were trained to consume a semipurified diet within a 1.5-hour period daily. Fasting plasma triglycerides were elevated significantly in the germfree animals. Analysis of the fatty acid composition of the fasting plasma triglycerides showed a higher percentage of linoleic acid in the germfree group. Postprandial plasma triglyceride analysis indicated a faster rate of clearance of the absorbed triglyceride by the germfree rat but did not indicate any apparent difference in the rate of absorption of the dietary triglyceride. Fasting plasma glucose levels were found to be elevated in the germfree animals. Glucose tolerance tests showed that the germfree rats clear glucose from the plasma more slowly than conventional rats. Immunoassay of plasma insulin obtained during the glucose tolerance testing indicated a delayed and decreased insulin secretion by the germfree rats.

It is well-known that a close relationship exists between the host and its microflora. The physiology, morphology and biochemistry of the germfree animal differs from that of the conventional animal in many respects (1). The presence of a microbial flora produces structural changes in the small intestine. Compared with the germfree animal, the small intestine of the conventional animal shows increases in weight, hydration and reticuloendothelial cell population. It has been suggested (2) that the decreased lamina propria tissue and cellular tissue associated with the defense mechanism leaves the mucosal surface of the germfree intestine more efficient for absorption; for example, thiamine disappears faster from the intestinal tract of the germfree animal.[4] Xylose absorption, as measured by both in vivo and in vitro techniques, is increased in germfree rats and mice (3).

Only a few studies have been concerned with lipid metabolism in the germfree animal. Plasma cholesterol level in the germfree rat is lower than in the conventional animal (4) and is accompanied by a decrease in bile acid turnover (5). Carcass fat has been reported to be greater in the conventional rat than in the germfree (6). These data suggest that the microflora might influence the absorption and metabolism of lipids; the present paper reports such a study in the germfree rat as compared with conventional control animals.

MATERIALS AND METHODS

Germfree and conventional male rats of the Fisher strain [5] were reared in this laboratory. The animals were housed in pairs and had access to water at all times. The maintenance of a germfree environment requires the steam sterilization of the diet and therefore the semipurified diet used in these experiments (table 1) was designed

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[4] Gordon, H. A., E. B. Kardoss and D. Kan 1960 Effects of normal microbial flora on structural and absorptive characteristics of the intestine. Abstracts 5th International Congress of Nutrition. Federation of American Societies for Experimental Biology, Washington, D. C., p. 13.

[5] Charles River Breeding Laboratories, North Wilmington, Massachusetts.

TABLE 1
Composition of experimental diet

	%
Casein	20.0
Amylopectin [1]	52.0
Cellulose powder [2]	10.0
Salts-L II [3]	5.0
B-Vitamin mixture-75 [3]	0.8
L-Cystine	0.2
Corn oil	11.85
Vitamins A, D, E, K [3]	0.15

[1] CoFlo Starch, National Starch Co., New Orleans, Louisiana.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Zimmerman, D. Z., and B. S. Wostmann 1963 Vitamin stability in diet sterilized for germfree animals. J. Nutr., 79: 318.

to make allowances for destruction of heat-labile components after exposure to steam. The dietary fat, corn oil, and the solution of fat-soluble vitamins were introduced directly into the germfree isolator by filtration through 0.22-μ pore size membrane filter [6] under positive nitrogen pressure to prevent oxidation of these labile components during steam sterilization. The remainder of the diet was steam sterilized and transferred into the isolator where the complete diet was mixed by hand.

Fifty-day-old animals of both experimental groups were trained to consume the diet daily within a 1.5-hour period. The controlled feeding permits a more consistent evaluation of blood lipids at fasting and postprandial periods. Using the combined chromatographic systems of silica gel glass fiber paper chromatography and gas-liquid chromatography (7) plasma triglyceride levels were determined using 10- to 25-μliter samples of fasting and postprandial plasma. The methyl esters of the triglyceride fatty acids were determined by gas-liquid chromatography using 6 mm × 180 cm glass coiled columns packed with 15% diethylene glycol succinate and a hydrogen flame detector. Identification of components was made with mixtures of methyl esters of fatty acids of known composition.

Blood samples were collected with heparinized capillary tubes from the suborbital capillary bed of animals which had been anesthetized with methoxyflurane.[7]

As a method of studying intestinal absorption of fatty acids more directly, linoleic-1-^{14}C acid was incorporated into the diet. Control animals were matched as to weight and pair-fed with the germfree animals. After consuming the test meal (about three-fourths of their normal daily consumption), the animals were placed in a closed system metabolism chamber (8) for a 2.5 hour period. CO_2-free air was circulated through the chamber and expired CO_2 was collected in trap containing 5 N NaOH. At the end of the metabolic experiment the animals were exsanguinated via cardiac puncture. Ligatures were applied at the pyloroduodenal junction and at the lower end of the ileum. The stomach and small intestine were removed and the contents of each compartment were washed out and collected; the liver was also removed for analysis. The gastric and intestinal contents were lyophilized and the lipids extracted in chloroform methanol (2:1, v/v); the liver was extracted by the same solvent. Aliquots of the lipid extract were added to scintillation fluid (9) and the radioactivity was measured in a liquid scintillation counter. Analysis of the expired CO_2 was made by placing aliquots of Na_2CO_3 on glass fiber paper, drying, adding to the scintillation fluid, and counting as above.

Glucose tolerance was determined 22.5 hours after the last feeding period by administration via stomach tube of 35% glucose solution (w/v) at a dosage of 1 ml/100 g of body weight. Blood samples were obtained as previously described at 45 minute intervals over a three hour period. Plasma glucose was determined by the glucose oxidase method.[8] Immunoreactive insulin levels were determined on these same samples using method C of Hales and Randle (10).

RESULTS

To obtain a baseline, fasting plasma triglyceride levels were determined on samples obtained 22.5 hours after the food cup was removed in both conventional and germfree rats and the data obtained are presented in table 2. The combined chromatographic techniques yield quantitative analysis of the triglycerides and both quali-

[6] Millipore Filter, Bedford, Massachusetts.
[7] Penthrane, Abbott Laboratories, North Chicago, Illinois.
[8] Glucostat, Worthington Biochemicals Corporation, Freehold, New Jersey.

TABLE 2

Fasting and postprandial plasma triglycerides and triglyceride fatty acid composition in germfree and conventional male rats

Sampling time	Status	Plasma triglyceride		Fatty acid				
				16:0	16:1	18:0	18:1	18:2
hours		*mg/100 ml*		%	%	%	%	%
Fasting	Conventional (13) [1]	44.2 ± 3.0 [2]	$P < 0.001$	33.2 ± 2.6	5.9 ± 1.1	11.9 ± 1.2	24.3 ± 1.2	24.3 ± 2.7
	Germfree (9)	122.9 ± 9.9		21.0 ± 1.4	4.4 ± 0.1	7.5 ± 1.0	28.6 ± 0.1	36.5 ± 0.8
Post-prandial 2.5	Conventional (10)	308.0 ± 11.4	$P = < 0.01 > 0.001$	19.0 ± 1.6	2.9 ± 0.2	3.6 ± 0.6	28.1 ± 0.7	46.1 ± 2.6
	Germfree (7)	225.0 ± 17.9		18.2 ± 1.7	1.8 ± 0.1	5.0 ± 0.7	28.9 ± 1.1	46.0 ± 2.4
4	Conventional (8)	170.9 ± 15.6		22.4 ± 1.7	5.6 ± 0.2	6.6 ± 0.2	20.0 ± 0.2	40.1 ± 2.1
	Germfree (7)	183.1 ± 11.3		17.7 ± 1.8	2.9 ± 0.1	7.3 ± 1.8	29.6 ± 0.2	42.6 ± 3.7
6	Conventional (8)	93.5 ± 7.5		28.2 ± 2.0	3.1 ± 0.6	8.6 ± 1.8	26.9 ± 1.1	31.5 ± 2.4
	Germfree (7)	141.9 ± 8.6		23.7 ± 1.5	4.8 ± 0.8	8.1 ± 0.8	24.7 ± 1.5	37.0 ± 1.2
12.0	Conventional (7)	84.7 ± 5.7		25.9 ± 2.1	4.3 ± 0.8	7.1 ± 1.0	29.7 ± 2.0	33.0 ± 2.1
	Germfree (8)	125.8 ± 5.1		25.0 ± 1.9	5.0 ± 0.6	9.4 ± 1.0	27.5 ± 0.8	33.2 ± 3.0

[1] Numbers in parentheses indicate number of animals.
[2] Values are means ± SE.

tative and quantitative analysis with respect to the component fatty acids. Plasma triglycerides are significantly ($P = < 0.001$) increased in the germfree as compared to the conventional rat. Analysis of the fatty acid composition of triglycerides shows an increase in linoleic acid in the germfree group with a compensating decrease in palmitic acid.

Following removal of the food cups, postprandial blood samples were taken at intervals over a 22.5-hour period and analyzed for triglyceride levels; the data obtained are also presented in table 2. In both the conventional and germfree groups, a peak plasma triglyceride level was observed 2.5 hours after removal of the food cups. However, the level of triglycerides in the conventional animals (which is lower at fasting-time) is now much greater, as compared with the level observed in germfree animals.

Further examination of the data shows that the postprandial plasma triglyceride level in germfree rats returns to the fasting level by the twelfth hour, while the levels observed in the conventional group are still slightly elevated at this time.

In table 3 the changes in the composition of the plasma triglycerides that occur during the first 2.5 hours after the feeding ends are shown. The increase in triglyceride concentration suggests a slower rate of triglyceride absorption by germfree animals. The fatty acid composition of the triglyceride added to the blood during this interval is similar for both groups of animals but is different in that no palmitoleate or stearate was added to the blood of germfree animals.

At the later time-intervals of 4 and 6 hours the the triglyceride level in the germfree animals remained elevated, which further suggests impaired clearance of absorbed dietary fat or increased synthesis of triglyceride by the liver.

The fate of radioactivity following feeding of the diet with linoleic-1-^{14}C acid is shown in table 4. There appears to be no detectable effect of the intestinal microflora on the absorption of linoleate as indicated by the amount of label remaining in the stomach and small intestine after 4 hours. The appearance of more radioactivity in the expired carbon dioxide from

TABLE 3

Change in concentration of plasma triglycerides observed 2.5 hours postprandially

Status	Plasma triglyceride	Fatty acid				
		16:0	16:1	18:0	18:1	18:2
	mg/100 ml	*mg/100 ml plasma*				
Conventional (13) [1]						
Fasting	44.2± 3.0 [2]	14.6±1.1	2.6±0.5	5.2±0.5	10.9±0.5	10.6±1.2
2.5 hours postprandial	308.0±11.4	60.1±4.9	10.5±0.7	16.7±1.8	86.5±2.2	143.9±8.0
Increase	264.8	45.9	7.9	11.5	75.6	133.3
		% of total				
Fractional increase		16.7	2.9	4.2	27.8	48.6
		mg/100 ml plasma				
Germfree (9)						
Fasting	122.9± 9.9	25.7±1.7	5.4±0.1	8.9±1.3	35.2±0.1	45.4±1.0
2.5 hours postprandial	225.0±17.9	43.8±3.8	4.5±0.3	7.1±1.6	61.7±2.5	105.9±5.4
Increase	102.1	18.1	—	—	26.5	60.5
		% of total				
Fractional increase		17.3	—	—	25.2	57.5

[1] Numbers in parentheses indicate number of animals.
[2] Values are means ± SE when SE is given; other values are the means.

TABLE 4

Individual recovery of radioactivity after feeding an experimental diet containing linoleic-1-^{14}C acid [1]

Animal	Stomach contents	Contents of small intestine	Liver	Expired CO_2	Total
	%	%	%		%
Germfree	56.4	0.6	21.8	12.8	90.6
	55.3	0.5	20.8	15.1	91.7
Conventional	51.0	0.8	21.1	21.7	94.6
	50.3	0.7	23.6	21.6	96.2

[1] Ten microcuries of linoleic acid 1-^{14}C were dissolved in corn oil and blended into the diet. The amount of fatty acid consumed was determined by subtracting the amount of radioactive fatty acid recovered from the food cup from the amount added to the diet; see text for other details.

the conventional animals suggests a greater rate of oxidation of the newly absorbed fatty acid although there was no observable difference in the amount of linoleate noted in the liver.

A glucose solution was administered by gastric tube to animals of both groups and blood samples were collected at intervals during the following 3-hour period. As shown in figure 1, the plasma glucose level of the germfree animal rises to a higher level and remains elevated up to 3 hours as compared with that of the conventional animal which returns nearly to normal in 3 hours. The increase in plasma glucose level in the conventional animal is not as marked as that of the germfree animal.

These plasma samples obtained in the experiment above were assayed for circulating immunoreactive insulin and these data are presented in figure 2. The data suggest that the conventional rat rapidly releases insulin-like activity and that a higher level of immunoreactive insulin is observed at 45 and 90 minutes following the administration of the glucose meal.

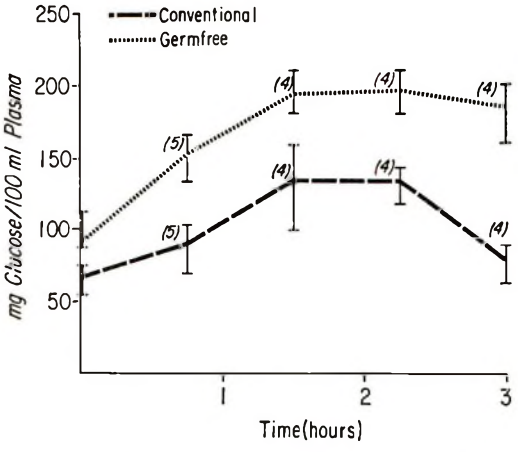

Fig. 1 Average glucose tolerance curves are shown for conventional rats and germfree rats. The vertical lines represent the range of individual values. Number of samples are given in parentheses.

Later, however, in the glucose tolerance test, the germfree animal has the higher level of immunoreactive insulin. The peak level of immunoreactive insulin in the conventional animal is reached more quickly after a stimulus from the glucose meal, suggesting that insulin release is delayed in the germfree animal.

DISCUSSION

The studies by Gordon (2) showed that the small intestine of the germfree rat has poorly developed lymphoid tissue. It has been suggested that the decreased thickness of the lamina propria leaves the mucosal surface of the germfree intestine able to absorb more efficiently. The appearance of dietary lipid in the peripheral blood is the resultant of the rate of absorption of lipid from the lumen of the intestine and the rate of removal of the absorbed lipid by the peripheral tissues and is used to evaluate the absorption of lipid. Fasting plasma triglyceride levels were established in germfree and conventional young adult male rats. The fasting triglyceride level for the germfree animals was approximately threefold higher than that observed in conventional rats. These data were unexpected since there was no reason to suspect a hyperlipemia; another lipid, serum cholesterol had been reported to be decreased in the germfree rat (5). The elevated fasting plasma triglyceride level of the germfree rat was due to increased levels of all of the glyceride component fatty acids; however, the content of linoleic acid in the germfree group was higher than any other of the fatty acids.

Analysis of the postprandial plasma triglyceride samples from both the germfree and conventional animals showed a peak triglyceride level at the end of 2.5 hours following the completion of the feeding period. The increased level of plasma tri-

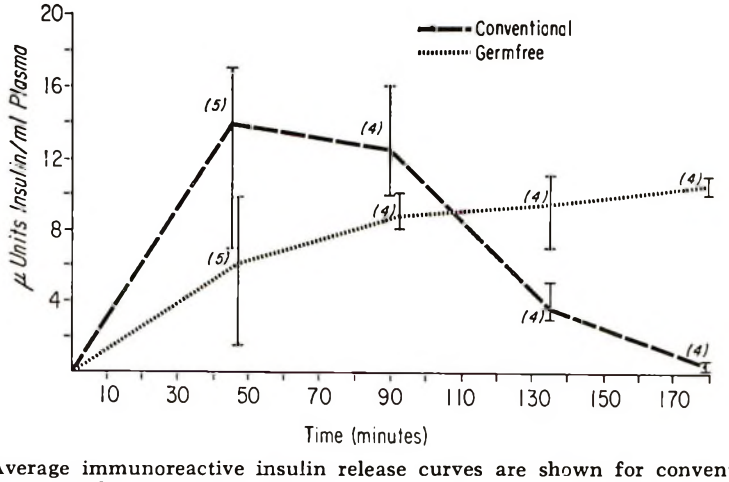

Fig. 2 Average immunoreactive insulin release curves are shown for conventional rats and germfree rats. These data were obtained on the same samples used for the data shown in figure 1. The vertical lines represent the range of individual values. Number of samples are indicated in parentheses.

glycerides at this time was much greater in the conventional group than that observed in the germfree group, even though the conventional group started from a lower fasting level. Further examination shows that the postabsorptive level of triglyceride in the germfree rats returns to the fasting level earlier than in the conventional rats. To explain the diminished response of plasma triglycerides in the germfree rats 2.5 hours postfeeding and the faster return to the fasting level, the following considerations are examined: 1) if absorption occurs earlier in the germfree rats and the rate of clearance of the absorbed lipid is the same, the germfree group would elicit a peak-plasma level earlier than the conventional rats and would also return to the fasting level at a faster rate; 2) however, if absorption is delayed in the germfree rats and the rate of clearance by the peripheral tissues is the same for both groups, then the conventional rats would show an earlier rise in plasma triglycerides and return sooner to the fasting level; and 3) when absorption is the same for both groups, the peak-plasma triglyceride levels would occur simultaneously. A faster clearance of the absorbed lipid under the latter circumstance would be demonstrated by a faster return to the fasting level. As a method for studying the rate of absorption, linoleic-1^{14}C acid was incorporated into the experimental diet and fed to animals of both groups. The percentage of radioactivity retained in the stomach and lumen of the small intestine for 2.5 hours postfeeding showed that the amount of lipid absorbed was about the same for both the germfree and conventional animals. Therefore, it appears likely that the difference observed between the germfree and conventional rats was not the result of impaired lipid absorption but rather a difference in the rate of clearance of the newly absorbed lipid. The decreased appearance of $^{14}CO_2$ in the germfree animal as compared with the conventional animals suggests that there is a decreased oxidation of linoleic acid. This decrease in oxidation of linoleic acid might account for the increased linoleate in the fasting plasma triglycerides of the germfree rats. Because of the slightly larger amount of label remaining in the stomach of the germfree animal, as compared with the conventional animal, this hypothesis should be studied with larger groups of animals or with in vitro studies. If, however, this effect were to result of gastric retention, reduced absorption and hence reduced oxidation, similar reduction in radioactivity content of the liver would have been anticipated. This was not observed.

The occurrence of carbohydrate intolerance associated with hyperglyceridemia was reported by Waddel et al. (11). It was noted that patients with elevated plasma triglyceride levels also had abnormal glucose tolerance. According to Vaughn (12) insulin is necessary for the storage of fatty acids by adipose tissue and that insufficient amount of tissue reactive insulin accelerates fatty acid mobilization from the adipose tissue with a resultant increase in glyceride formation by the liver. The fasting plasma glucose levels of the germfree rats were elevated above the levels observed in the conventional rats. The germfree animal exhibited a greater rise in plasma glucose during a glucose tolerance test, and a much slower return to the fasting level as compared with the conventional animals. The glucose tolerance of the conventional rat was found to be similar to that described by Uram et al. (13), whereas the glucose tolerance curve obtained from the germfree rat appeared to demonstrate intolerance to glucose. This apparent intolerance was accompanied by a slower release of immunoreactive insulin into the plasma. Three hours after administration of the glucose, the plasma level of immunoreactive insulin in the conventional rat was extremely low which coincided with a low plasma glucose level and a high triglyceride level. However at this time, the level of immunoreactive insulin in the germfree rat was elevated and the plasma glucose levels were only beginning to return to the fasting level. These data indicate that the germfree animal has difficulty clearing glucose from the circulation and that plasma immunoreactive insulin in these animals was released more slowly than in the conventional animal. The evidence presented implies that the fasting hyperglyceridemia observed in the germfree rat may be secondarily related to a

carbohydrate intolerance, and in this sense the germfree rat appears to be similar to a diabetic rat. However, other contributing factors may also be acting concertedly to produce the hyperglyceridemia.

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Trace Metals in Rat Tissues as Influenced by Calcium in Water [1]

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ABSTRACT To ascertain whether calcium in drinking water inhibited accumulation of several trace metals in tissues, 40 Long-Evans weanling rats were separated as to sex and into groups, half receiving 200 ppm calcium. The diet, of rye, skim milk, and corn oil, was high in calcium. The water contained: (ppm) chromium, 2; manganese, 10; nickel, 5; copper, 5; zinc, 50; cadmium, 5; and lead, 5. At 400 to 533 days of age tissues were analyzed for these trace metals. No significant effect of calcium on mean concentrations in 5 organs was found. Renal cadmium, copper and zinc levels were highly correlated. The incidence of hypertension was partly suppressed in female rats rceiving calcium in drinking water. Hypertensive animals of both sexes had more extractable aortic lipids than did normotensive rats. Serum cholesterol levels were unaffected. Apparently calcium in drinking water, comparable to that in "hard" potable water did not affect the deposition in tissues of trace metals contained in the water.

A statistical relationship between the degree of softness of potable water and death rates from hypertension or atherosclerotic heart disease has been demonstrated repeatedly (1–4). The possibility exists that this relationship might be concerned with trace elements in water and their accumulation in tissues (3). Rats given small amounts of cadmium in drinking water have regularly developed arterial hypertension after a year or more (5, 6). Therefore, rats were studied to ascertain whether an increment of calcium in drinking water, such as might be found in "hard" water, affected the accumulation or deposition of chromium, manganese, copper, zinc, nickel, cadmium and lead in the water. The incidence of hypertension and the concentration of lipids in the aortas were also measured, as having possible bearing on this question.

METHODS

Twenty male and 20 female rats of the Long-Evans (hooded) strain were born in this laboratory and littermates separated into 2 groups at the time of weaning, four to a cage. One group was given drinking water containing soluble trace metal salts and 200 ppm calcium as the chloride (here designated as "hard" water for convenience); the other group was given the water with the trace metals without calcium ("soft" water). The water used came from a forest spring, was doubly deionized and had a conductivity of 3 to 5 million ohms.

The laboratory was especially built to avoid metallic contamination; air was electrostatically precipitated (7). The diet was composed of 30% dry skim milk, 60% locally ground rye flour, 9% corn oil and 1% sodium chloride, with added vitamins and iron, as reported (7). Content and approximate intakes of trace and bulk metals in the drinking water and the diet are shown in table 1. The diet was high in calcium, mainly from the milk.

When a rat from one group died, his counterpart from the other group was killed by ether and the livers, kidney, hearts, lungs and spleens were frozen in separate polyethylene bottles. It was necessary to pair organs from each group to obtain sufficient tissue for analysis. The earliest death occurred at 395 days, the next at 409 days and three subsequent ones at 440 to 488 days, all in males. Surviving rats were killed in pairs at intervals of 505 to 533 days of age.

Systolic blood pressure was measured by the method of Friedman and Freed (8),

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TABLE 1

Estimated intakes of trace and bulk metals by rats

Metal	Concentration		Approximate intake		Total
	Water	Food	Water [1]	Food [2]	
	μg/g	μg/g	μg/100 g body wt/day		
Chromium	2	0.1	14	0.6	14.6
Manganese	10	8.1	70	48.6	118.6
Cobalt	1	0.4	7	2.4	9.4
Copper	5	3.3	35	19.8	54.8
Zinc	50	22.3	350	139.8	489.8
Molybdenum	1	— [3]	7	— [3]	7.0+
Nickel	5	0.4	35	2.4	37.4
Cadmium	5	0.02	35	0.12	35.1
Lead	5	0.2	35	1.2	36.2
Calcium [4]	0 [5]	4128	0	24,768	24,768
Calcium [4]	200 [6]		1400		26,168
Magnesium	0	902	0	5412	5,412

[1] Based on 7 g H_2O/100 g body weight.
[2] Based on 6 g food/100 g weight/day.
[3] Amount in food undetermined.
[4] Based on analyses in literature (22).
[5] Amount without added calcium.
[6] Amount with added calcium.

using a microphone on the tail; rats were anesthetized with sodium pentobarbital, 45 mg/kg body weight, given intraperitoneally (5). At autopsy, aortas were examined under ultraviolet light for the presence of spontaneous plaques, and lipids were extracted with petroleum ether according to a method published previously (9), and expressed as milligrams/100 g dry weight. Serum cholesterol was measured by a pre-mixed reagent [3] according to the method of Huang et al. (10).

Tissues were treated in accordance with established methods (11) to avoid contamination, and ashed in muffle furnaces at 450°. Analyses for chromium, nickel, copper, zinc and cadmium were made by atomic absorption spectrophotometry,[4] for manganese and lead by the methods of Sandell (12). Practical limits of detection of the elements in solutions of ash and in standard solutions, in micrograms per milliliter and percentage reproducibility of the methods at working concentrations in tissues according to 10 repetitive analyses were, respectively: chromium, 0.01 μg and 10%; nickel, 0.05 μg and 4%; copper, 0.01 μg and 4%; zinc, 0.01 μg and 2%; cadmium, 0.01 μg and 5%; manganese, 1.0 μg and 5%; lead, 0.5 μg and 2%.

More than 1,000 analyses were made. Because of the small size of most of the organs, and the amount required for analysis, each pair was analyzed once for manganese and lead and twice for the other elements. In the case of liver, multiple analyses were possible. Data were analyzed by Student's *t*, chi-square, or coefficients of correlation (13), as applicable.

RESULTS

Tissue concentrations. The individual deposition of the 7 trace metals in the 5 tissues analyzed was usually quite variable despite relatively constant intakes. The mean concentration in each organ of rats taking hard and soft water did not differ significantly (tables 2 and 3) at these levels of intake. Because concentrations in male tissues were similar to those of females, the data were combined. The wide ranges made statistical analyses meaningless in most cases.

Predilection of certain metals for certain tissues was evident. Chromium in spleen, copper in kidney, zinc in liver and kidney, nickel in heart and spleen, cadmium in kidney and liver were noted in higher concentrations than in other organs (tables 2 and 3). The ranges of chromium concentration in heart and of copper and cadmium concentration in kidney were outside the ranges in some other organs, and their means differed significantly ($P < 0.01$).

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[4] Perkin-Elmer 303, The Perkin Elmer Corporation, Norwalk, Connecticut.

TABLE 2

Influence of calcium in water on the deposition of chromium, manganese, copper and zinc in rat tissues [1]

Organ	Type of water	Chromium	Manganese	Copper	Zinc
		μg/g	*μg/g*	*μg/g*	*μg/g*
Liver	hard [2]	0.66 ± 0.129 [3]	2.30 ± 0.812	5.95 ± 0.912	44.2 ± 8.43
Liver	soft [4]	0.72 ± 0.170	1.48 ± 0.436	5.61 ± 1.140	39.4 ± 8.39
Kidney	hard	1.63 ± 0.313	1.55 ± 0.583	34.06 ± 8.920	46.8 ± 10.62
Kidney	soft	1.41 ± 0.238	1.32 ± 0.403	28.05 ± 5.821	48.2 ± 10.08
Lung	hard	1.39 ± 0.228	1.32 ± 0.433	6.14 ± 0.895	24.8 ± 3.95
Lung	soft	1.01 ± 0.201	1.24 ± 0.296	9.44 ± 3.712	29.2 ± 7.64
Heart	hard	2.18 ± 0.220	2.32 ± 1.441	13.82 ± 3.59	18.8 ± 3.02
Heart	soft	—	2.22 ± 0.953	18.50 ± 6.72	22.7 ± 6.13
Spleen	hard	3.55 ± 1.596	1.55 ± 0.706	15.41 ± 7.34	20.4 ± 6.75
Spleen	soft	2.64 ± 1.075	1.88 ± 0.706	14.61 ± 2.73	25.5 ± 3.92

[1] Nine to ten analyses were made on each sample composed of tissues from 2 rats.
[2] Containing calcium 200 ppm.
[3] Means ± SEM, wet weight.
[4] Without added calcium.

TABLE 3

Influence of calcium in water on deposition of nickel, cadmium and lead in rat tissues

Organ	Type of water	Nickel	Cadmium	Lead	Ash
		μg/g	*μg/g*	*μg/g*	*% dry wt*
Liver	hard [1]	0.23 ± 0.021 [2]	6.7 ± 2.29	0.33 ± 0.083	4.7 ± 0.14
Liver	soft [3]	0.38 ± 0.065	5.9 ± 1.23	0.25 ± 0.069	4.5 ± 0.32
Kidney	hard	0.86 ± 0.203	42.8 ± 14.61 [4]	0.74 ± 0.246	6.6 ± 0.54
Kidney	soft	0.80 ± 0.103	43.1 ± 8.82	1.22 ± 0.383	6.2 ± 0.33
Lung	hard	0.85 ± 0.121	0.9 ± 0.14	0.74 ± 0.152	7.3 ± 0.49
Lung	soft	0.80 ± 0.178	0.6 ± 0.15	0.78 ± 0.181	7.1 ± 0.61
Heart	hard	2.78 ± 0.652	1.0 ± 0.21	1.17 ± 0.619	8.3 ± 1.37
Heart	soft	2.15 ± 0.265	0.9 ± 0.20	0.85 ± 0.229	8.5 ± 1.00
Spleen	hard	2.32 ± 0.736	0.9 ± 0.13	0.92 ± 0.314	9.8 ± 0.35
Spleen	soft	2.30 ± 0.296	0.9 ± 0.20	0.55 ± 0.267	8.5 ± 0.60

[1] Containing calcium 200 ppm.
[2] Means ± SEM wet weight and on ash, % dry weight.
[3] Without added calcium.
[4] Mean renal ratios Cd:Zn, hard 0.85 ± 0.137, soft 1.03 ± 0.128; ratios of Cd:Zn of control rats were 0.016 or less.

The concentrations of copper, cadmium and zinc in kidney and liver were related, when the groups taking hard and soft water were combined (table 4). Correlations of other metals were not apparent from the data. No relations of tissue metals to age of the animals were found between 400 and 533 days of age.

The percentage of ash in the various tissues of the 2 groups did not differ significantly. Mean ash percentage of wet weight was almost identical for liver, lung and heart. Rats given soft water had 0.23% less ash in kidney and 0.25% less ash in spleen, but the mean differences were not statistically significant. On a dry-weight basis, all organs except heart had slightly more ash when animals were given calcium (table 3).

Physiological functions. In table 5 are indicated various data on the rats receiving hard and soft water. No significant differences between the 2 groups appeared for body weight (or growth rates), intake of water, the proportion with spontaneous aortic plaques, serum cholesterol or the mortality. Extractable aortic lipids were less in calcium-fed animals, significantly so in females.

The incidence of hypertension in the 4 groups is shown in table 6. Normotensive and hypertensive animals were sharply divided, there being no overlapping of their ranges of blood pressure. All females and 60% of males receiving the soft water exhibited hypertension, whereas only 3 animals given hard water were hypertensive. Aortas of hypertensive animals of both

TABLE 4

Paired rank correlations of concentrations of trace metals in rat kidney and liver

	Liver		Kidney	
	Copper	Zinc	Copper	Zinc
Zinc	+0.33 [1]	—	+0.74 [2]	—
Cadmium	ns [3]	+0.49 [4]	+0.70 [2]	+0.95 [5]

[1] $P < 0.1$.
[2] $P < 0.005$.
[3] Not significant.
[4] $P < 0.025$.
[5] $P < 0.001$.

TABLE 5

Means of data on rats given cadmium, nickel and lead with and without calcium in drinking water

	Without calcium	With calcium
Males [1]		
Wt, g, 365 days	449.4	463.7
Water intake, g/100 g body wt/day	7.0	7.07
Aortic plaques found, %	30	50
Extractable aortic lipids, mg/100 g	197.8 ± 16.2 [2]	159.6 ± 28.6
Serum cholesterol, mg/100 ml	85.6 ± 2.3	88.4 ± 2.4
Dead, 500 days, %	20	30
Females [1]		
Wt, g, 365 days	298.2	296.2
Water intake, g/100 g body wt/day	6.73	7.07
Aortic plaques found, %	0	0
Extractable aortic lipids, mg/100 g	319.4 ± 67.2	161.5 ± 14.5 [3]
Serum cholesterol, mg/100 ml	97.0 ± 8.9	94.3 ± 6.1
Dead, 500 days, %	0	0

[1] Ten animals/group.
[2] Means ± SEM.
[3] Differs from no-calcium group, $P < 0.02$.

TABLE 6

Mean blood pressures of rats given cadmium, nickel, and lead with or without calcium in water

Type of water	Normotensive			Hypertensive		
	No. rats	Mean	Range	No. rats	Mean	Range
		mm Hg	*mm Hg*		*mm Hg*	*mm Hg*
Males						
Hard [1]	6	98	83–109	1	260+	
Soft [2]	4	85	76–90	6 [3]	242	220–260+
Females						
Hard [1]	8	92	65–127	2	260+	
Soft [2]	–			10 [3]	253	192–260+

[1] Containing calcium 200 ppm.
[2] Without added calcium.
[3] Chi-square difference from hard water groups of both sexes combined, 11.9 ($P < 0.001$).

sexes combined contained 275.5 ± 47.1 mg/100 g of extractable lipids, whereas those of normotensive rats contained 142.8 ± 12.1 mg/100 g ($P < 0.001$).

DISCUSSION

It is apparent that at these levels of intake of calcium, which were only 5.3% higher in rats receiving hard rather than soft water, no effect on deposition of metals in tissues was observed. Therefore, calcium in drinking water did not influence the tissue deposition of the metals contained in the water. The percentage of the total calcium intake supplied in water to rats was identical to that estimated for human

beings in 25 hard water areas, that is, 5.3% (4).

High intakes of calcium in food have produced deficiencies of copper in sheep, of manganese and zinc in pigs and chicks, and of iron, zinc and iodine in rats (14) on low intakes of these elements. Overt signs of trace metal deficiency were not observed in these rats, whether given additional calcium or not, probably because their intakes were adequate.

In only 2 cases was there a suggestion that rats given soft water contained more metal in their tissues than those given hard water, but the trends were not uniform. In males mean nickel was higher in liver ($P < 0.01$) but not in females, and females had higher values in kidney ($P < 0.05$) but not males.

The variations in the tissue concentration of the essential trace metals, chromium, manganese, copper and zinc (table 2), were probably not the result of methodology, for all tissues were treated alike. Similar variations have been found in human tissues by emission spectroscopy (15–17), which were presumed to result from differences in intake. The percentage ash of dry tissue varied to a lesser degree than in human tissues, where variations were usually threefold or more (15). These variations lead to the conclusion that factors inherent in the individual animal govern the tissue concentration of these essential trace elements at the moment of death, and suggest that their turnover may be rapid in the tissues examined.

The association of renal and hepatic cadmium and zinc concentration has been demonstrated in man (18, 19) and is here shown in rats. The high coefficient of correlation for the 2 metals in kidney indicates that as cadmium is deposited, zinc is also bound. Apparently as the ratio of cadmium to zinc increases, some biochemical process, as yet unknown, effects a rise in arterial pressure (6). A similarly high cadmium-to-zinc ratio has been observed in human beings dying of the consequences of hypertension (20).

The increase in aortic lipids in the female rats given soft water was probably a result of hypertension (5) and not of cadmium alone. We have reported increased aortic lipids in rats given cadmium (9); normotensive animals in this present series had less aortic lipids than hypertensive ones, whether or not they were given calcium. Whereas the effect of calcium on partly suppressing hypertension appears clear in female animals, it is difficult to account for the results in terms of tissue deposition of trace metals.

We have previously reported excessive mortality in male rats and mice from these intakes and at these tissue concentrations of both lead and cadmium given separately (10), and no increased mortality in mice given nickel (21). Presumably the early mortality of male rats in this present series could be accounted for on the basis of lead and cadmium toxicity. Their tissue concentrations are well within the range of human values (16). In a series of 104 control rats studied concurrently, which were receiving the essential trace elements without cadmium, lead or nickel, the mortality was 4 and 6% at 500 days of age in males and females, respectively. Tissue concentrations of cadmium and lead in another series of control rats have been reported (11).

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Long-term Rat Feeding Study with Used Frying Fats

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ABSTRACT A study was made to learn whether fats which had been exposed to the heat and aeration of actual frying differ significantly from fresh fats in their nutritional properties. Partially hydrogenated soybean oils, cottonseed oil, and lard were used for frying under practical restaurant-type frying conditions until they became unfit for further use owing to excessive foaming during frying. The used fats were fed to groups of 50 male and 50 female rats as 15% of the diet for 2 years. The used fats were slightly less absorbable than unheated control fats, and gave correspondingly slower growth rates. Other than this there were no differences in clinical, metabolic, or pathological criteria to suggest that the used fats adversely affected the rats consuming them. Mortality among the heated-fat groups was no higher than among the control groups. Distillable non-urea-adductable fractions concentrated from the used fats proved somewhat toxic when large doses of them were administered by stomach tube to weanling rats. The results indicate that, although heating of fats under actual frying conditions does cause the formation of substances which can be shown to be toxic, the level of such substances and the degree of their toxicity are so low as to have no practical dietary significance.

Fatty materials undergo chemical reactions when they are strongly heated in air. Evidence has been offered that some of the reaction products which form under certain heating conditions are toxic when fed to rats (1, 2). It seemed important to learn whether fats which had been exposed to the heat and aeration of actual frying differ significantly from fresh fats in their nutritional properties.

Of the publications reporting toxicity in heated fats, some (3–5) have dealt with fats which had been heated under extreme conditions of temperature, or aeration, or both. Others (6, 7) report the concentration of small quantities of toxic materials from large volumes of heated fat. And in still others it appears that manifestations ascribed to fat toxicity should have been ascribed to oxidative deterioration of improperly stored mixed diets (8–11). The significance of these results as they relate to human nutrition is not clear.

Work has been reported in which fats that actually had been used for restaurant and commercial frying were evaluated nutritionally by short-term feeding studies and were found to be unimpaired in value (12, 13). The long-term feeding studies which have been reported used fats that had been oxidized to high peroxide value at low temperature (14), or polymerized at high temperature in the absence of air (15), or bubbled with air during heating (16). In each case no ill effects were observed in the animals that consumed these fats, but, since the conditions used for preparing the fats differed considerably from practical frying conditions, these experimental results also are not directly applicable to practical human nutrition.

In a recently published study, Lanteaume et al. (17) fed rats with diets containing 15% of heated fats. The fats were grapeseed oil which had been used 60 times for frying potatoes without replenishment of fat lost by absorption in the potatoes, or grapeseed oil which had been heated 2 days or 4 days in an open beaker at 200°. The feeding study started with rats 7 months of age and lasted for one year. Unheated grapeseed oil was slightly more absorbable than the fat used for frying and gave slightly greater weight gains. The artificially heated fats were slightly less absorbable, and gave slightly lower weight gains. Otherwise no significant differences were observed among the groups according to the several clinical, biochemical, and histological observations made.

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We wished to conduct an experiment whose results, more nearly than any of those cited above, would demonstrate whether fats repeatedly used in actual frying practice have any meaningful effects in normal diets. For this experiment we studied 7 fats. Two of these were unheated controls. Four had been used for frying until they reached the end of their frying usefulness as evidenced by excessive foaming tendency. One was used for frying until it had attained a steady state of foaming tendency. The distillable non-urea-adducting fractions of these fats were isolated and bioassayed, and the fats themselves were fed to rats over a 2-year period.

EXPERIMENTAL

Fats. The fresh and used fats studied in this experiment are described in table 1. The table also shows the code letter assigned to each fat, which will be used to identify it in the other tables. All the fats had been prepared by normal factory or pilot plant processes. The cottonseed oil and soybean oils had been refined, bleached, and deodorized. Analyses of the fats are reported in table 2.

Frying procedure. The used fats were prepared by heating them in 60-kg (fat capacity) gas-fired kettles. The fats were heated at 182° each weekday from 8 AM to 4 PM. At 9 AM and at 2 PM in each kettle 3.95 kg each of frozen potatoes, breaded scallops, and onion rings, in that order, were fried for 5 minutes, 2.5 minutes, and 3 minutes, respectively. These foods were obtained through normal commercial food channels. At 4 PM daily 2.47 kg of fat were added to each kettle to replace the fat that had been absorbed by the fried foods. The kettles were allowed to cool overnight and on weekends. During the frying of the onion rings, the height of foam was measured above the previously measured level of still fat in the kettle. When the foam height exceeded 75 mm that fat was removed from the kettles and protected against oxidative changes which might have occurred during storage by the addition of 39 ppm butylated hydroxytoluene plus 31 ppm butylated hydroxyanisole. The fats were sealed in 453-g cans under nitrogen before storage in a frozen-food warehouse. Fat D (soybean oil, IV 108, with silicone) never attained the 75-mm foam height end point owing to the stabilizing effect of the silicone. Frying in that fat was discontinued after it became apparent that a steady state of foam height had been reached due to fat turnover.

TABLE 1

Fats studied

Code	Description
A	Soybean oil, fresh (not used for frying)
B	Soybean oil, hydrogenated to IV 108, fresh
C	Soybean oil, hydrogenated to IV 108, used for frying
D	Soybean oil, hydrogenated to IV 108, with 1.6 ppm methyl silicone added, used for frying
E	Soybean oil, hydrogenated to IV 70, used for frying
F	Cottonseed oil, used for frying
G	Lard, used for frying

Total duration of the heating ranged from just over a week to over 5 weeks. Table 2 shows the number of hours each of the fats was heated and the analytical values after heating. Samples of used fats were withdrawn from storage and analyzed at intervals up to 2 years; the zero- and 2-year analyses are also shown in the table.

Feeding study. The fats were incorporated at a level of 15% into the semipurified diet shown in table 3. The diets were prepared fresh each week and kept refrigerated until dispensed into the feeding cups. Feeding was carried out 3 times per week, and any feed remaining in the cups was discarded, so that the longest period that any of the feed was unrefrigerated after mixing was 3 days. Previous experiments (unpublished) had shown that the results obtained with such a system of diet handling were indistinguishable from results obtained with daily mixing of diets, whereas weekly feeding had led to relatively poor growth with diets containing used fats.

Four hundred each of male and female rats [3] were received as weanlings. After 3 days the extremely light and heavy animals were discarded. The remainder were sorted into 7 groups of 50 males and 50 females, balanced on the basis of weight and litter. The rats were kept individually

[3] Simonsen Standard Laboratory, Sprague-Dawley-originated, Caesarean-derived rats from Simonsen Laboratories, White Bear Lake, Minnesota 55110.

TABLE 2

Analyses of fats

Fat	Frying time	Storage time	Lovibond color [1]	Free fatty acid [1]	Iodine value [1]	Peroxide value	n_D^{60}	Polar fraction [2]	Conjugated diene [3]	Conjugated triene [3]	Fatty acid composition by gas-liquid chromatography						
											14:0	16:0	16:1	18:0	18:1	18:2	18:3
	hours	*years*		%				%	%	%	%	%	%	%	%	%	%
A	0	0	10/1.0	0.04	129.0	1.6			50.6	7.3	0	11	0	5	27	51	6
	0	2	12/1.2	0.03	130.0	0.5	1.4603	1.4	51.2	7.7	0	11	0	4	26.5	52	6
B	0	0	3/0.3	0.02	108.2	1.2	1.4578		33.5	3.0	tr	11	0	5	45	35	4
	0	2	4/0.4	0.02	108.5	0.1	1.4577	1.3	33.2	3.1	0	11.5	0	6	46	34.5	2
C	0	0	3/0.3	0.02	108.2	1.2	1.4578		33.5	3.0	0	12	0	6	50	31	1
	60	0	300/80	0.65	100.5	3.8	1.4597	13.6	28.1	2.3	0	13	0	6.5	50	30	0.3
	60	2	300/80	0.76	101.0	3.5	1.4597	14.6	28.1	2.3							
D	0	0	3/0.3	0.02	108.5		1.4578				tr	11	0	5	46	35	3
	216	0	3500/430	8.10	101.5	0	1.4589	30.2	33.6	3.0	0	11	0	6	50	32	1
	216	2	1800/400	8.20	101.5	0.7	1.4588	32.5	28.5	2.7	0	12	0	6.5	49.5	30.5	1.5
E	0	0	3/0.2	0.03	69.7	0	1.4533		5.1	0.5	0	15	0	12	66	7	0
	84	0	300/60	1.30	63.5	5.5	1.4552	20.1	3.2	0.08	tr	16	0	14	65	5	tr
	84	2	300/80	1.30	66.4	1.9	1.4551	19.0	2.6	0.1	0	16	0	14.5	65.5	4.0	0
F	0	0	8/0.8	0.03	109.0	0.6	1.4581		53.8	0.44	1	27	0	2	17	53	0
	49	0	200/50	0.45	102.5	4.6	1.4601	14.2	44.9	0.33	1	31	0	2	17	48	0
	49	2	200/60	0.48	102.0	4.4	1.4598	15.2	46.5	0.6	1	30	0	2.5	17	49.5	0
G	0	0	1/0.1	0.05	64.4	0	1.4527		13.1	0.98	1	24	0	13	46	10	3
	116	0	600/110	2.30	55.6	3.0	1.4550	23.4	7.5	0.42	1	30	0	15	46	6	0
	116	2	500/140	2.40	55.7	1.3	1.4550	25.5	7.5	0.40	2	31	1.5	15	44.5	5.5	0

[1] AOCS method (24)

[2] Measured by adsorption chromatography on silicic acid containing 4% methanol; eluted with methanol:benzene 1:49. Method of E. N. Frankel (unpublished).

[3] AOCS method (24) involving alkali isomerization and UV absorption, as linoleate and linolenate.

TABLE 3

Composition of diet

	%
Casein [1]	5.0
Non-fat dry milk [2]	21.0
Ground whole wheat	43.0
Dried egg white [3]	3.0
Dried liver, defatted [4]	3.0
Vitamin mixture in sucrose [5]	3.0
L-Lysine·HCl	0.5
Calcium phosphate, dibasic [6]	1.0
Salt mixture [7]	3.5
Vitamin mixture in soybean oil [8]	2.0
Experimental fat	15.0
Kilocalories/g	4.4

[1] Labco, Vitamin-Free, Whitson Products Division of the Borden Company, New York.
[2] Land O'Lakes Creameries, Minneapolis.
[3] Nutritional Biochemicals Corporation, Cleveland; biotin was added at a level of 1 mg/100 g of albumen.
[4] Wilson Laboratories, Inc., Chicago.
[5] Furnished the following in mg/kg of diet: menadione, 1.8; thiamine, 2.4; riboflavin, 3.0; niacin, 12.0; folic acid, 0.15; Ca pantothenate, 12.0; pyridoxine, 2.4; inositol, 12,000; p-aminobenzoic acid, 60.0; biotin, 0.015; cyanocobalamin, 90.0; ascorbic acid, 60.0; and choline chloride, 1800.
[6] Added to maintain calculated Ca and P at 2% and Ca:P ratio at 1.1:1.0.
[7] Phillips and Hart (25) but with the addition of 0.005% cobalt chloride; obtained from Nutritional Biochemicals Corporation, Cleveland.
[8] Soybean oil furnished essential fatty acids, and the mixture furnished the following IU per kg of diet: 12,000 vitamin A; 3,200 vitamin D_2; and 100 vitamin E as *d*-*α*-tocopheryl acetate.

in galvanized steel cages, 26 × 30 × 17 cm, at 23 ± 1° and 50 ± 5% relative humidity. They received 12 hours of light per day uniformly. The individuals within the groups were so distributed as to avoid bias caused by position within the racks or the room. Feed and water were given ad libitum. Feed consumption records were maintained for each animal. Each week the animals were weighed and carefully examined for abnormalities. As they aged, sick ones were separated from the rest, and their values were removed from the subsequent growth and feed data, but were included in the longevity and pathology data.

Metabolic studies. For the determination of fat absorbability, and coefficient of absorbability, feces were collected from 10 randomly selected rats per sex per dietary group for two weeks at 2, 12, and 21 months, by means of wire-screen collectors fastened below the regular cages. The feces were dried in vacuo at 80°, cleaned, and pulverized. Total fatty acids were determined by saponification with alcoholic KOH, acidulation, dilution with water, extraction with petroleum ether, drying and evaporating the extract, and weighing the dried residue. Fecal nitrogen was determined by the Kjeldahl method. For the calculations, endogenous fat was assumed to be 0.03 g/day for males and 0.02 g/day for females; these values were based on our experience with similar animals fed purified rations.

Urine was collected from 10 randomly selected animals per sex per group at 2, 12, and 21 months. The rats were given water but not feed during the 12-hour collection period, which followed a 12-hour fast. Rats were kept in metabolism cages for the collection. The urine was collected under toluene; each sample was diluted to 25 ml and analyzed for nitrogen (Kjeldahl), and for sugar, albumin, bilirubin, and ketone bodies by means of test tablets and strips. [4]

Clinical studies and pathology. From each group and sex there were chosen at random 5 rats at 12 months and 10 rats at 24 months. Blood samples were obtained from these animals by caudal artery puncture for blood cell counts, hematocrit, hemoglobin, and blood glucose analyses. The same animals were anesthetized with ethyl ether, and 5-ml samples of blood were drawn from the heart for plasma cholesterol (18) and plasma phospholipid determinations (19). The animals were then put to death by excess of the ether; the heart, liver, and kidneys were removed and weighed for the calculation of organ-to-body weight ratios. The heart, liver, and kidneys were assayed for sodium, potassium, and calcium by flame photometry at 12 months and by atomic absorption spectroscopy at 24 months. Liver cholesterol and phospholipid values were determined. Peritoneal fats were converted to methyl esters, and their fatty acid compositions were determined by gas-liquid chromatography. Thymus, heart, lung, liver, stomach, pancreas, spleen, adrenal, kidney, mesenteric lymph nodes, ileum, gonads, and any apparent neoplasms were removed for histological examination. Slides were prepared and studied within our laboratories, and were also examined by a consulting veterinary pathologist. Animals that died or were killed because moribund were au-

[4] Ames Company, Inc., Elkhart, Indiana.

topsied, and if putrescence had not started the same tissues were removed for similar histology.

Statistical treatment. Where applicable the analysis of variance was used to test for significance. The final growth and feed consumption data were tested by the use of harmonic means because of the extreme disproportionalities produced in the data by intermediate deaths (20). Minimum significant differences were computed by the method of Tukey as described by Scheffe (21).

Isolation and bioassay of distillable non-urea-adducting fractions (DNUA). The DNUA's were isolated by a modification of the method of Crampton (22). A 1-kg portion of each fat was saponified with aqueous-alcoholic KOH. The soaps were acidulated, and the free fatty acids were extracted. The acids were converted to ethyl esters by twice refluxing for 30 minutes with 2 volumes of ethanol containing 0.5% H_2SO_4. The esters were distilled under high vacuum at pot temperatures up to 240°. Each distillate was mixed with 4 times its weight of powdered urea and 4 times its volume of ethanol. The slurry was held at 55° for 30 minutes before cooling overnight to room temperature. After filtration the filter cake was washed with urea-saturated alcohol. The combined alcohol solutions were mixed with water and extracted with hexane, then ether. The extracts were concentrated, dried, and evaporated under reduced pressure, leaving behind the DNUA. Yields of DNUA are shown in table 4.

For bioassay 0.5 ml of a DNUA was administered daily for 3 consecutive days to each of 5 weanling male rats (7). Administration was by stomach tube. The rats were given a commercial pelleted ration and water ad libitum during the experiment. Survivors were killed on the tenth day, at which time body and thymus weights were determined. As controls, similar groups of rats were not given any DNUA, or else were given the DNUA from olive oil which had been heated 50 hours at 182°, the latter having previously been shown in our laboratory to be toxic under these conditions. Results of the bioassay are shown in table 4.

RESULTS

Fats. Analytical values on the fresh fats (table 2) showed them to be typical of their respective kinds. The analytically measurable changes which took place in the fats during frying were generally similar to the changes observed by many other

TABLE 4
Acute toxicity test

Sample characteristics [1]	Level of DNUA in fat	Avg body wt gain [2]		Thymus wt [3]	Diarrhea incidence	Mortality
		3 days	10 days			
	% by volume	*g*	*g*	*mg*	*%*	*%*
DNUA of Fat A	2.0	17.2	69.4	425	0	0
DNUA of Fat B	1.5	19.6	71.2	446	0	0
DNUA of Fat C	2.1	0.3	54.7	359 (81)	0	40
DNUA of Fat D	2.0	9.2	59.6	391	0	0
DNUA of Fat E	2.0	−10.4	24.0	167 (32)	100	20
DNUA of Fat F	2.3	7.8	55.6	328	0	0
DNUA of Fat G	1.7	9.8	57.3	409 (88)	0	20
Positive control [4]	2.4	−10.0	39.3	142 (84)	40	40
Negative control, pellets only	—	21.6	66.6	445	0	0

[1] There were 5 weanling male rats/group, and each rat was given 0.5 ml of lipid/day for 3 consecutive days. DNUA = distillable non-urea-adductable fraction.
[2] These results are for surviving animals only.
[3] Weights shown are averages for animals living at 10 days. Values in parentheses are for dead rats; normal rats of the same age as the dead ones have thymus weights averaging 210 mg.
[4] DNUA of olive oil heated 50 hours at 182°

TABLE 5

Cumulative growth, feed consumption, and feed efficiency [1]

Fat	Avg wt gain			Avg feed consumption			Feed efficiency [2]			Coefficient of absorbability [3]		
	2 months	12 months	21 months	2 months	12 months	21 months	2 months	12 months	21 months	2 months	12 months	21 months
	g	*g*	*g*	*g*	*g*	*g*	%	%	%	%	%	%
						Male						
A	365 ^{a}	727 ^{a}	783 ^{a}	974 ^{a}	6619 ^{ab}	11870 ^{a}	37.4 ^{a}	11.0 ^{a}	6.6 ^{a}	96.2 ^{a}	96.5 ^{a}	97.0 ^{a}
B	358 ^{ab}	725 ^{ab}	763 ^{a}	963 ^{a}	6511 ^{a}	11747 ^{ab}	37.2 ^{a}	11.1 ^{a}	6.5 ^{a}	95.5 ^{a}	96.8 ^{a}	96.6 ^{a}
C	339 ^{bc}	677 ^{b}	764 ^{a}	951 ^{a}	6435 ^{a}	11674 ^{ab}	35.7 ^{b}	10.5 ^{abc}	6.5 ^{a}	90.3 ^{b}	92.4 ^{b}	94.2 ^{b}
D	344 ^{abc}	704 ^{ab}	774 ^{a}	965 ^{a}	6604 ^{ab}	11848 ^{ab}	35.6 ^{b}	10.6 ^{ab}	6.5 ^{a}	91.5 ^{b}	92.7 ^{b}	92.2 ^{b}
E	340 ^{bc}	703 ^{ab}	747 ^{a}	1001 ^{a}	6929 ^{b}	12292 ^{ab}	34.0 ^{b}	10.1 ^{bc}	6.0 ^{b}	82.2 ^{c}	88.2 ^{c}	86.9 ^{c}
F	339 ^{bc}	682 ^{ab}	727 ^{a}	962 ^{a}	6506 ^{a}	11861 ^{ab}	35.3 ^{b}	10.5 ^{abc}	6.1 ^{ab}	87.9 ^{d}	90.0 ^{d}	89.8 ^{d}
G	335 ^{c}	679 ^{ab}	737 ^{a}	976 ^{a}	6855 ^{b}	12380 ^{b}	34.7 ^{b}	9.9 ^{c}	6.0 ^{b}	87.8 ^{d}	89.4 ^{cd}	89.3 ^{d}
						Female						
A	200 ^{de}	405 ^{c}	525 ^{b}	732 ^{b}	4897 ^{c}	9409 ^{c}	27.5 ^{d}	8.2 ^{a}	5.6 ^{a}	96.8 ^{e}	97.5 ^{e}	97.6 ^{e}
B	204 ^{d}	392 ^{c}	518 ^{b}	726 ^{b}	4825 ^{c}	9238 ^{c}	28.0 ^{d}	8.1 ^{ab}	5.6 ^{a}	96.4 ^{e}	96.8 ^{e}	96.4 ^{e}
C	196 ^{de}	375 ^{c}	466 ^{b}	731 ^{b}	4901 ^{c}	9164 ^{c}	26.9 ^{de}	7.6 ^{bc}	5.0 ^{ab}	92.1 ^{fg}	92.7 ^{fg}	92.5 ^{fg}
D	201 ^{de}	377 ^{c}	463 ^{b}	744 ^{b}	4974 ^{c}	9323 ^{c}	27.1 ^{d}	7.6 ^{bc}	4.9 ^{ab}	92.7 ^{f}	94.0 ^{f}	93.6 ^{f}
E	189 ^{e}	362 ^{c}	446 ^{b}	746 ^{b}	5018 ^{c}	9262 ^{c}	25.5 ^{f}	7.1 ^{c}	4.8 ^{b}	87.6 ^{h}	89.6 ^{h}	89.6 ^{h}
F	196 ^{de}	374 ^{c}	486 ^{b}	741 ^{b}	4913 ^{c}	9145 ^{c}	26.4 ^{ef}	7.6 ^{bc}	5.3 ^{ab}	91.1 ^{gi}	91.8 ^{g}	92.0 ^{g}
G	196 ^{de}	372 ^{c}	454 ^{b}	749 ^{b}	5131 ^{c}	9441 ^{c}	26.1 ^{ef}	7.2 ^{c}	4.8 ^{b}	89.8 ^{i}	91.9 ^{g}	90.3 ^{h}

[1] Any 2 values within a column which are followed by the same letter are not significantly different ($P \geqq 0.05$) from each other.
[2] Feed efficiency = weight gain × 100/feed consumption.
[3] For experimental fat level only. Allowance was made for fat-soluble vitamin mixture and metabolic fat.

workers in the past. There were small but irregular increases in the free fatty acid levels, peroxide values, and refractive indices, and small decreases in the iodine values. There were small decreases in the relative levels of polyunsaturated fatty acids, presumably as a result of oxidation and polymerization. The content of polar material in each fat increased markedly during frying. The ultraviolet, gas chromatographic, and urea adduction results show that the majority of the fatty acid chains were unaffected by the heating procedures. The properties of the fats did not change appreciably during storage for 2 years, showing that the storage conditions were adequate for preservation of the fats in their originally prepared states.

Two-year feeding study. Table 5 shows mean growth and feed consumption data for 2, 12, and 21 months. Values for intermediate times were generally similar to the ones shown, and are omitted for brevity. The 24-month growth data are omitted since deaths among the senescent animals caused extreme variations within the groups. During the early, rapid growth stage the two fresh fats produced generally more rapid growth than the others, although Fat D (soybean oil, IV 108, with silicone) was nearly equivalent. The growth differences were more pronounced among the male than the female rats. The differences in body weights which arose during the early growth period tended to persist throughout the experiment, although they became relatively smaller with time, and statistically insignificant with increasing variance among the individuals. Part of the reason for the diminution of weight differences with time was that the heavier groups, namely those receiving Fats A (fresh soybean oil), B (fresh soybean oil, IV 108), and D (used soybean oil, IV 108, with silicone), had slightly higher mortality rates than the others, and these deaths occurred mostly among the more obese individuals. Deaths of the more obese animals in the heavier groups lowered the mean weights of those groups.

The differences in growth could not be attributed to differences in feed consumption. Instead, rapid growth appears to have been associated with high feed efficiency, and is accounted for by the values given for coefficient of absorbability (table 5). Two factors appear to have influenced absorbability: heating, and degree of unsaturation. The unheated fats were most absorbable, and also, on account of the experimental design, the most unsaturated. Among the heated fats, the 2 samples of lightly hydrogenated soybean oil (C and D) were more absorbable than the lard or cottonseed oil, while Fat E, the soybean oil which had been hydrogenated to IV 70 before frying, showed the lowest values for coefficient of absorbability. This same relationship of absorbabilities prevailed in both sexes at all ages. The lower absorbability of the heated fats is probably due to their content of unabsorbable (22) polymeric materials, formed during heating.

Detailed results of the many clinical analyses carried out — fecal nitrogen, urine volume, pH, nitrogen, and protein, blood counts, hemoglobin, hematocrit, blood glucose, plasma cholesterol and phospholipid, organ-to-body weight ratios, liver lipid, phospholipid and cholesterol, liver and kidney calcium, sodium, and potassium — are not shown in the tables. The values were generally within normal ranges and were not remarkable. Many of the values show differences between the sexes and at different ages, but there were few statistically significant ($P = 0.05$) differences assignable to dietary groups within the age-sex groupings. All of the statistically significant differences which were seen are summarized in table 6. Most of these differences appear to be of a random nature, showing no pattern which can be correlated with fresh vs. used fats. An exception to this is the liver cholesterol-to-phospholipid ratio at 24 months; the relatively high values observed in the male groups receiving unheated fats might be related to the relatively high incidence of obesity in those groups. The biological significance of differences in mineral content of the various organs is questionable, since these values showed wide variations, both within and among the various groups. Analyses of peritoneal fats showed their fatty acid compositions to be generally parallel to the fatty acid compositions of the respective dietary fats.

Longevity and pathology. Table 7 shows the percentage of animals surviving at

TABLE 6

Statistically significant differences in metabolic and clinical values

Analysis	Sex	Age	Direction of difference
		months	
Fecal nitrogen	F	21	B > A, D, G
Urine volume	F	12	E > A, C, D, F
Heart wt/body wt	M	12	C > E
Liver wt/body wt	F	12	E > A, B, D
Liver wt/body wt	F	24	F > A
Liver cholesterol	M	24	A > B, C, D, E, F, G
Liver cholesterol/phospholipid	M	24	A, B > C, D, E, F, G
Kidney calcium	F	12	F > D
Liver potassium	F	24	A, B, C > D, E, F, G
Kidney potassium	F	12	G > C
Kidney potassium	F	24	A, C > E
Kidney sodium	F	24	A > B, D, F, G

TABLE 7

Percentage of animals surviving at various ages

Diet group	Males, age in months				Females, age in months				Total, both sexes
	12	18	21	24	12	18	21	24	
A	96	84	80	62	92	80	60	33	48
B	98	87	73	53	100	91	80	58	56
C	96	96	87	67	98	91	80	60	63
D	88	73	66	53	98	89	80	58	55
E	100	87	82	69	96	89	80	67	68
F	100	96	82	64	100	93	87	56	60
G	98	93	91	62	98	96	93	84	73
Avg	96.5	88	80	61	97	90	80	59	60

several ages. Sixty percent of the animals survived for 2 years. There was only a slight difference between the sexes in most groups. Survival rates were slightly lower for animals receiving the two unheated fats and Fat D (soybean oil, IV 108, with silicone, used) than for animals receiving the other heated fats. This difference parallels and is attributed to the larger mean weights, the higher incidence of obesity, and the better utilization of fat in those same groups. The table shows what appears to have been a high incidence of early mortality in the males receiving Fat D (soybean oil, IV 108, with silicone, used). The value shown resulted from 6 deaths within the first year; two were from unknown causes, one from a tumor of the prostate, two from pneumonia, one from a bladder infection. The pneumonia and bladder infection are not uncommon among such animals as these in our laboratory, and we see no reason to suppose that the distribution of mortality was not random.

Table 8 shows the incidence of tumors, respiratory disease, nephritis, and liver pathology observed grossly in all rats. The females developed more tumors than the males, owing to their susceptibility to mammary carcinoma. The higher incidence of tumors in the females contributed to their having mortality rates equal to the males. No diet-related differences in tumor incidence appeared in the males, but the females receiving the two fresh fats had a higher incidence of tumors than those receiving used fats. It has been reported (23) that a direct relationship exists between caloric intake and tumor risk in the rat, and, as pointed out above, the two fresh fats furnished more absorbable energy than the used fats.

The histological findings on the 10 rats killed from each group are summarized in table 9. In both sexes the incidence of

TABLE 8

Percentage incidence of various pathological conditions [1] (all animals, all ages)

Fat	A		B		C		D		E		F		G	
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Tumors [2]	20.0	55.6	22.2	53.3	17.8	37.8	22.2	28.9	22.2	33.3	26.7	28.9	13.3	31.1
Respiratory disease [3]	22.2	11.1	15.6	6.7	22.2	13.3	20.0	11.1	11.1	8.9	2.2	6.7	11.1	6.7
Nephritis	30.0	8.0	24.0	14.0	20.0	12.0	22.0	6.0	30.0	12.0	18.0	12.0	30.0	18.0
Liver pathology	24.0	34.0	48.0	38.0	6.0	28.0	18.0	24.0	28.0	30.0	12.0	26.0	22.0	30.0

[1] Grossly observable tumors and disease symptoms only.
[2] Male tumor incidence, 20.6%; female tumor incidence, 38.4%.
[3] Severe respiratory disease with clinical symptoms; male respiratory disease incidence, 14.9%; female respiratory disease incidence, 9.2%.

fatty infiltration of the liver was relatively high in animals receiving the unheated fats and quite low in animals receiving Fat E (soybean oil, IV 70, used) or Fat G (lard, used). Thus it appears that this condition also may be related to the intake of absorbable energy, but not specifically to the ingestion of fresh or used fats. None of the other frequently seen pathology appears to have been related to dietary differences. Various other pathological conditions were observed only infrequently, and could not be related to dietary differences.

Acute toxicity test. Crampton et al. (22) showed that the toxic substances formed by heating linolenate under carbon dioxide could be concentrated into the DNUA fraction. The distillable urea-adducting fraction contained esters of unchanged fatty acids, while the non-distillable fraction contained polymers which, although not nutritious, were non-toxic by reason of their non-absorbability. For assaying the presence of toxic substances in the DNUA fractions of heated fats, we have used a method (7) which involves forced feeding of weanling rats with large quantities (ca. 30 g/kg) of the DNUA over a 48-hour period. Our experiences with this procedure show that its results parallel those observed by Crampton, but it is quicker and uses much less material. Table 4 shows that, as expected, DNUA fractions from the fresh fats did not harm the rats receiving them, while those from the heated fats were all harmful in one or more respects, causing diminished weight gains, premature involution of the thymus, diarrhea, or death. The number of animals used was too small to permit quantitative comparisons among the various used fats; it is clear that all of them contained low levels of substances toxic to the weanling rat.

DISCUSSION

The aim of this experiment was to feed high levels of fats which had been heated under conditions as severe as any ever likely to be encountered as constituents of fried foods in the normal American diet. Therefore we chose what we believed to be the worst possible conditions for preparing the fats, short of conditions which

TABLE 9

Percentage incidence of pathology in two-year-old rats (histological examination of ten rats/group, per sex)

Fat	A		B		C		D		E		F		G	
Sex	M	F	M	F	M [1]	F	M	F	M	F	M [1]	F	M	F
Fatty liver	40	50	30	20	22	10	20	20	0	—	11	10	10	—
Chronic pyelonephritis	50	50	80	50	77	20	80	30	60	10	55	50	40	50
Tubule mineralization in kidney	—	40	—	20	—	40	—	60	—	50	—	30	—	30
Adrenal telangiectasis	—	30	30	70	22	70	—	30	10	20	—	40	20	40
Alveolar foam cells	20	10	20	—	—	—	20	10	20	30	66	20	10	20
Tumors:														
Mammary	—	60	—	70	—	40	—	20	—	30	—	30	—	20
Others	20	—	10	20	33	0	20	20	10	—	22	—	30	10

[1] Only 9 slides/group were examined by the consulting pathologist.

would have been wholly unrealistic in relation to culinary practice.

Of the ways in which fats are used in cooking, three involve relatively severe heat treatment. We may distinguish these as 1) pan frying, 2) continuous frying, and 3) intermittent deep-fat frying. In pan-frying or sautéing, fats are heated strongly, but only for short periods of time. Such fats are not ordinarily saved and re-used, and hence there is little opportunity for any transformation products which might form to build up to substantial levels.

In many commercial frying operations, such as doughnut or potato chip manufacturing, where frying is continuous, fat is constantly absorbed by the food and carried out, to be replaced by fresh fat. The turnover time is short enough so that there is little opportunity for significant changes in fat composition to occur; the levels of color, flavor, foaming tendency, and free fatty acids are not objectionable, so it is seldom or never necessary to discard the fat and replace it with new.[5] It seems clear that if one is to look for nutritionally undesirable changes in used frying fat, they should be sought not here, but rather in the low fat-turnover operations of small volume, batch-frying establishments.

Some restaurants frequently keep their fat hot for many hours a day, fry in it occasionally, allow it to cool overnight, and reheat it next day. While this pattern of use continues, the polymer content and viscosity of the fat increase. Eventually it either foams over the sides of the kettle or becomes unacceptably dark in color; then it is discarded and replaced. (In many restaurants, of course, the frying volume is great enough so that there is a rapid turnover of fat, and here the situation is not unlike that which exists in the continuous frying operations discussed above.) In our opinion, those fats used for frying until they threaten to foam out of the kettle are the most severely heated fats likely to be consumed in the normal human diet, and it was such fats that we chose for our feeding study. Along with them we fed a fat (D) whose frying life had been greatly extended through the use of silicone, and which had been used for frying until its content of transformation products (as indicated by foam height) appeared to have reached a steady state through fat turnover, even though the quantity of food being fried in it was relatively small.

Most previous workers have studied fats which had been heated at higher temperatures or with more vigorous aeration than would be found in actual frying practice. Attempts to apply conclusions based on

[5] Robertson, C. J. 1966 The principles of deep fat frying for the bakery. Bakers Dig., 40 (5): 54.

such work to human nutrition seem unwarranted, owing to the likelihood that such artificially abused fats may differ from practically used fats in the kind, as well as the levels, of transformation products which they contain.

Some factor of exaggeration is necessary and appropriate in feeding studies for detecting mild toxicity or low levels of toxic substances. Such exaggeration was achieved in the acute bioassay stage of our work by concentrating the altered fatty acids from used fats through urea adduction. The DNUA fractions of the used fats elicited manifestations of acute toxicity, and even caused death in some cases, when 1.5-ml doses of them were given to weanling rats over a 48-hour period. It is clear from this that toxic substances did form in our fats during the heating program, although at quite low levels. To administer in whole fat the same quantity of these toxic substances as was administered in the DNUA concentrate would have required dosing each rat with more than his own body weight of used fat during a 48-hour period.

Chronic toxicity of the DNUA's and of other used fat components were evaluated in the long-term stage of our experiment. In the 2-year study, a considerable degree of exaggeration compared with human diets was maintained (a) by using the fats up to the practical end-point of their usefulness for frying, rather than to some intermediate point corresponding to the average of the fats being used in actual food preparation, (b) by feeding them at the high level of 15% of the diet, and (c) by feeding them steadily throughout the lifetime of the animals. Even with these exaggerations, the experiment produced no evidence that used frying fats adversely affected the health or longevity of the animals consuming them.

The literature reports that artifically abused fats contained substances which are toxic to laboratory animals. From such reports it has been inferred that it may be harmful for humans to consume used frying fats. We have found that actual used frying fats contain only very small quantities of substances which are toxic when administered in large doses to weanling rats, and that the fats themselves produce no appreciable ill effects on animals consuming them.

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Protein Quality of a Soybean Protein Textured Food in Experimental Animals and Children [1,2]

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ABSTRACT Studies were carried out in experimental animals and children to evaluate the protein quality of a textured food simulating ground beef, and made from isolated soybean protein with added egg albumin and wheat gluten. On the basis of a PER value of 2.50 for casein, the PER of the textured food and of natural dehydrated beef was 2.30 and 2.34, respectively. Highest weight gain was obtained with diets containing 16.7 and 16.3% protein, from the soybean protein textured food and casein, respectively. NPU values were 62.6 for casein and 59.1% for the soybean protein textured food. Heating of the soybean protein textured food increased weight gain but not the PER. Heating of the protein isolate and of the fiber made from it improved both. Apparently this treatment caused the elimination of adverse physiological factors inherent in soybean, or the removal of substances in the product derived from the preparation process. At the 10% protein level, supplementation with lysine and methionine added together, but not alone, improved protein quality. Growth and nitrogen balance studies with dogs indicated that the soybean protein textured food had essentially the same protein quality as that of dehydrated beef. True protein digestibility and biological value were 92.3 and 65.3%, respectively, for the soybean protein textured food, and 87.0 and 67.4% for the dehydrated beef. The results in children show that, at a protein intake level of 2 g/kg/day, no difference in quality was evident between skim milk and the soybean protein textured food. Nitrogen equilibrium was obtained when the children received approximately 138 mg of nitrogen from the soybean protein textured food, as compared with 97 mg from milk. The true protein digestibility and biological value was 92.3 and 65.3%, respectively. It was concluded that the protein quality of the soybean protein textured food was about 80% of that from milk. It was readily accepted by the children and free of adverse physiological effects.

Highly purified proteins are being isolated now from oil-free, food-grade protein concentrates such as soybean, cottonseed and sesame. Among these, protein isolates prepared from oil-free soybean flakes have received the greatest attention. The protein content of the isolate runs as high as 95%, and the products are bland in taste and have none of the flavors normally associated with the flours and other similar products (1–3).

The protein isolates are available in monofilament, granular or powder forms, which make them suitable for a wide range of functional uses, such as whipping, emulsifying, gelling, stabilizing, thickening and moisture-binding. Thus, the number of food products which can be made from them is practically unlimited.[3-6]

The essential amino acid pattern present in such isolates is, in the majority of cases, essentially the same as that in the material from which it was prepared. However, the process of isolation, eliminating certain protein fractions, as well as the use of variable temperatures, treatment with chemicals and pH changes, may alter the nutritive value of such products. Information on the nutritive value of the protein of these isolates is not very extensive, and

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[2] INCAP Publication I-428.
[3] Boyer, R. A. 1954 High protein food product and process for its preparation. Cincinnati, Ohio. U. S. Patent 2,682,466 (issued June 29).
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[5] Anson, M. L. and M. Pader 1959 Method of preparing a meat-like product. Assignors to Lever Brothers Company, New York, U. S. Patent 2,879,163 (issued March 24).
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there is even less material or possibly none at all on textured foods prepared from them.

The purpose of the work reported in this paper was, therefore, to evaluate the protein quality of a ground meat-like soybean protein textured food, in experimental animals and children.

MATERIALS AND METHODS

Soybean protein textured food.[7] The soybean protein textured food used in the biological studies to be described was obtained from General Mills, Inc. Commercially available protein isolates prepared from soybean meal, when dispersed in aqueous alkali, display pseudo-plastic characteristics. Such a viscous "dope," when forced through textile spinnerets into a suitable coagulation bath, is converted into continuous multiple monofilaments. Absence of hardening or chemical cross-linking agents permits their use as digestible structural elements for a new class of foods that are chewed. Suitable choice of flavoring, binding and other materials can give rise to products analogous to meat or other textured natural foods.

Protein suspensions for spinning were prepared using 16% of isolate solids in aqueous alkali of pH 12.0 at room temperature, with total alkaline exposure time of less than 10 minutes. Monofilament production was achieved by extrusion of the alkali suspension through platinum-rhodium alloy spinnerets into an aqueous bath of acetic acid, hydrochloric acid and sodium chloride, maintained at pH 3.5. The fibrils were subsequently rinsed free of precipitant and squeeze-dried before compounding. After thorough admixture of fibrils with all other formula ingredients, plus added water, the resultant mass was heated briefly to coagulate the albumin. The "beef" analogue so obtained was ground to granules and dried in hot air to less than 3% moisture content.

In this nutritional study, a simulated beef granule of the following dry weight percentage composition was used as test material: soybean protein fibrils, 28.8; vegetable fat,[8] 21.5; egg albumin, 12.3; wheat gluten, 11.8; toasted soybean meal, 9.6; vegetable protein hydrolysate, 1.7; brown sugar, 4.9; and non-nutritives (as flavoring, coloring), 9.4. In addition to the final product, the soybean protein isolate and spun fibrils made from it were also tested for protein quality per se in experimental animals. Samples were taken for chemical analysis before the feeding tests were begun. Aliquots of the isolate used and the resultant fibrils were withdrawn from the process stream and freeze-dried. Amino acid chromatography was carried out on the isolate, the fibrils and the dried finished product. The results are shown in table 1. Other proteins used for comparative purposes were vitamin-free casein and dehydrated beef. The dehydrated beef was round steak which was cut into small pieces and dried in an air convection oven at 70°. It was then ground to a powder, and at least 10 samples were

TABLE 1
Proximate composition and amino acid content (%) [1,2]

	Soybean protein		
	Isolate	Fibrils	Textured food
	%	%	%
Moisture	2.1	4.3	2.8
Ether extract	0.6	0.4	22.2
Crude fiber	0.5	0.7	1.3
Nitrogen	15.00	15.04	8.68
Protein (N × 6.25)	93.7	96.2	54.2
Ash	1.9	0.6	2.4
Lysine	5.41	5.12	3.22
Histidine	2.95	2.64	1.70
Ammonia	1.48	1.44	1.21
Arginine	7.67	7.85	4.54
Aspartic acid	9.85	10.51	6.12
Threonine	3.46	3.22	2.48
Serine	4.84	4.20	3.47
Glutamic acid	10.15	6.18	13.95
Proline	5.54	4.49	4.26
Glycine	3.08	2.93	2.10
Alanine	3.47	3.38	2.58
½ Cystine	0.84	0.72	0.96
Valine	3.98	4.22	3.16
Methionine	1.19	1.04	0.89
Isoleucine	4.58	4.46	3.28
Leucine	7.60	6.95	5.12
Tyrosine	3.62	3.62	3.08
Phenylalanine	5.45	5.12	3.97
Protein (N × 6.25)	95.3	96.9	56.4

[1] Proximate composition from INCAP laboratories on samples used for biological tests.
[2] Amino acid chromatographic analysis performed by the Department of Biochemistry, University of Minnesota, Minneapolis.

[7] Registered as "Bontrae," a generic name for General Mills, Inc., specialties of the spun protein class.
[8] Percentage fatty acid spectrum: myristic, 0.3; palmitic, 15.3; palmitoleic, 0.1; stearic, 9.9; oleic, 56.5; linoleic, 16.6; and linolenic, 1.4.

analyzed for proximate composition. All materials were kept under refrigeration at all times.

Biological tests with rats

The protein quality of the soybean protein textured food, of the isolate and of the protein fibers, was evaluated by several procedures, using rats. In all experiments weanling white rats of the Wistar strain, from the INCAP colony, were used. The number of animals used per experiment varied as indicated in the tables presenting the results. Each group consisted of equal number of female and male rats, distributed by weight so that the average initial weight was the same for all groups within an experiment. The animals were placed in individual all-wire screen cages with raised screen bottoms. They were fed ad libitum, and water was available at all times. For growth and PER studies, the changes in weight and in the amount of food consumed were recorded every week for a total of 28 days. All diets were analyzed for nitrogen content. In some experiments the animals were killed at the end of the growth period, and blood and liver collected for analysis. Protein evaluation with rats was carried out by a) feeding increasing levels of protein from the textured food, and calculation of PER; b) by the net protein utilization (NPU) assay, and c) by amino acid supplementation and PER calculation at a 10% protein level in the diet.

The NPU assay was carried out by feeding weanling rats for a 10-day period with the protein under study added to provide a 10% protein level in the diet. A group of rats was fed a nitrogen-free diet to correct for endogenous body nitrogen. At the end of the experimental period, the animals were weighed, killed, opened and placed in a hot-air oven at 80° for drying to constant weight. The whole dry carcass was ground in a micro-Wiley mill to pass 20 mesh. Duplicate samples were then taken for nitrogen analysis by the Kjeldahl method. NPU was calculated from the following formula (4):

$$\text{NPU} = \frac{\text{Carcass N test group} - \text{carcass N (N-free diet) group}}{\text{Dietary N intake}}$$

The composition of the basal diet (nitrogen-free diet) was: (in %) cottonseed oil, 11.72; cod liver oil, 1.00; mineral mixture (5), 4.00; dextrose, 25.00, and cornstarch, 58.28. All diets were supplemented with 5 ml of a complete B-vitamin mixture, per 100 g of diet (6). For the studies in which several levels of protein were fed, the soybean protein textured food, casein [9] and the dehydrated beef, replaced the cornstarch, and oil was added to keep the level of total calories constant (431 kcal/100 g). When amino acids were added, these also replaced part of the cornstarch. When the protein isolate and the fiber were tested in an experiment, these were included in the basal diet in amounts supplying 10% protein in the diet.

In some studies, the materials were cooked, even though the soybean protein textured food received had already been cooked. In this process the dried material was treated in the autoclave with an amount of water equal in weight, for 5 minutes, at 122.5°. After cooling all samples were freeze-dried, ground and stored under refrigeration until they were incorporated into the diets.

Biological tests with dogs

Growth studies. Twelve 2-month-old mongrel dogs, six of each sex, weighing between 1.6 to 2.7 kg, were used in these studies. The dogs were distributed into two equal groups according to weight and sex, one of which was fed the soybean protein textured food and the other dehydrated beef. The average initial weights of the 2 groups were practically the same (2.156 kg vs. 2.191 kg). The dogs from each group were fed approximately 9 g of protein/kg/day and 168 kcal/kg/day for 60 days. The soybean protein textured food and the dehydrated beef were incorporated into a diet containing: (in %) soybean protein textured food, 50.0 or dehydrated beef, 50.0: hydrogenated vegetable oil, 10.0; cod liver oil, 1.0; mineral mixture (5), 2.0; dextrose, 0.8; and dextrin, 29.0. The diet was also supplemented with 5 ml of a complete vitamin solution per 100 g (6). Each day, the amount of food to be offered the dogs was weighed

[9] Nutritional Biochemicals Corporation, Cleveland.

and suspended in water warmed at 38°. Records of weight changes were kept every 2 days.

Nitrogen balance. After completion of the experiment described above, the protein quality of the soybean protein textured food was studied by the nitrogen balance method. The animals were fed decreasing levels of protein per kilogram of body weight, from approximately 6 g to zero. During the last 12 days of the study, a nitrogen-free diet of the same composition described above was fed, except that the protein source was replaced by dextrin. The animals were weighed every 4 days to adjust for protein and calorie intake. The intake of calories was kept constant at 168 kcal/kg/day. Each level of protein was fed for 8 days divided into two 4-day balance periods. Feces and urine were collected daily and, at the end of each 4-day period, they were weighed or measured, homogenized and analyzed for total nitrogen content. The urine was collected in dark bottles containing 1 ml of concentrated acetic acid.

Nitrogen balance studies in children

Eight children between 22 and 72 months of age, and weighing between 9.03 and 16.25 kg, were used in these studies. These children entered the INCAP metabolism unit with protein-calorie malnutrition, but were in good health and completely recovered by the time they were placed on the protein evaluation study.

Each child was first fed whole milk for a 10-day period, to provide 2 g of protein/kg/day, followed by the feeding of the soybean protein textured food at decreasing levels of protein intake from 2 to zero grams of protein/kg body weight/day. Each level of protein was also fed for 10 days. The intake of calories remained constant at 100 kcal/kg/day. Vitamins [10] and ferrous sulfate (0.32 g/day) were added to each child's diet, in physiological amounts. The first 4 days of each 10-day period were used as an adaptation to the dietary change, and two 3-day balance periods were obtained from the remaining 6 days, in which feces and urine were collected quantitatively. An aliquot of the food consumed was also collected for nitrogen analysis every 3 days. Urine was collected in bottles containing 1 ml of concentrated acetic acid, which were constantly immersed in ice. The 3-day urine and fecal collections were weighed and homogenized before nitrogen analysis, which was performed by the macro-Kjeldahl method.

A representative composition of the daily food consumed both for the milk and the soybean protein textured food feeding is given in table 2 for case PC-164. To feed decreasing levels of protein, the soybean protein textured food was replaced by a mixture of dextrin, maltose and hydrogenated vegetable fat.

TABLE 2

Representative daily intake

	Milk [1]	Textured food [1]
	g	*g*
Whole milk	108	—
Sugar	70	80
Cornstarch	20	40
Mixture of dextrin and maltose	111	121
Margarine	2	—
Salt	1	—
Water	888	719
Soybean protein textured food	—	51
Hydrogenated vegetable fat	—	19
Tomato and onion flavoring	—	10
Salt	—	2
Water	—	1604

[1] A multivitamin preparation and $FeSO_4$ was given daily to the children in both diets.

RESULTS

Rats. The effect of feeding increasing levels of protein from the soybean protein textured food, from casein and from dehydrated beef is shown in table 3. At the 11.6% protein level in the diet, both protein sources gave maximum PER, with casein giving the highest, 2.66, as compared with 2.44 for the soybean product. On the basis of a PER of 2.50 for casein, the textured protein food would have a PER of 2.30, similar to that found for dehydrated beef.

As the protein content of the diet increased above 12%, PER decreased. The decrease was similar for all protein

[10] Each 0.6 ml provided 5000 IU vitamin A; 1000 IU vitamin D; 1 mg thiamine; 0.4 mg riboflavin; 1 mg pyridoxine; 2 mg panthotenic acid (no salt); 5 mg nicotinamide; and 50 mg ascorbic acid.

TABLE 3

Growth performance and other parameters of rats fed decreasing protein levels from the soybean protein textured food (SPTF), casein and dehydrated beef (12 rats/group)

Protein source	Amount in diet	Protein in diet	Avg wt gained [1]	PER	Serum proteins		Liver		
					Total	Albumin	Fresh wt	Moisture	Fat [2]
	%	%	g		%	%	g	%	%
SPTF	9.21	7.4	35 ± 15 [3]	1.58 ± 0.53	4.74 ± 0.28	2.44 ± 0.35	4.6 ± 3.8	65.4 ± 3.8	24.1 ± 10.7
SPTF	18.43	11.6	125 ± 21	2.69 ± 0.21	5.43 ± 0.37	3.04 ± 0.26	8.3 ± 1.4	66.9 ± 0.9	16.0 ± 4.5
SPTF	27.64	16.7	158 ± 22	2.28 ± 0.20	6.09 ± 0.38	3.18 ± 0.21	10.7 ± 1.7	67.0 ± 0.4	12.5 ± 3.3
SPTF	36.85	20.4	162 ± 34	2.04 ± 0.24	6.07 ± 1.00	3.10 ± 0.29	10.8 ± 2.5	67.6 ± 0.9	11.7 ± 3.4
SPTF	55.28	30.8	141 ± 30	1.32 ± 0.20	6.12 ± 0.36	3.11 ± 0.28	10.2 ± 1.7	68.2 ± 0.5	10.2 ± 1.6
Casein	5.60	7.2	29 ± 11	1.39 ± 0.53	4.78 ± 0.48	2.79 ± 0.27	4.5 ± 1.6	65.7 ± 3.8	32.4 ± 8.9
Casein	11.20	11.7	116 ± 18	2.66 ± 0.15	5.16 ± 0.42	2.92 ± 0.18	8.1 ± 1.0	67.4 ± 1.0	16.3 ± 3.7
Casein	16.80	16.3	165 ± 40	2.14 ± 0.30	5.81 ± 0.28	3.12 ± 0.20	10.6 ± 2.7	67.8 ± 0.7	13.9 ± 2.6
Casein	22.40	22.1	169 ± 37	1.91 ± 0.31	6.03 ± 0.29	3.33 ± 0.23	10.7 ± 2.3	68.0 ± 0.7	12.2 ± 1.9
Casein	33.60	31.4	170 ± 44	1.44 ± 0.22	6.03 ± 0.30	3.18 ± 0.11	10.9 ± 2.4	67.8 ± 0.8	11.5 ± 1.2
Dehydrated beef	8.31	8.4	51 ± 17	2.02 ± 0.38	—	—	—	—	—
Dehydrated beef	16.62	12.2	123 ± 22	2.66 ± 0.30	—	—	—	—	—
Dehydrated beef	24.93	17.0	151 ± 35	2.40 ± 0.29	—	—	—	—	—
Dehydrated beef	33.24	22.0	156 ± 34	1.94 ± 0.24	—	—	—	—	—
Dehydrated beef	41.55	27.5	166 ± 35	1.58 ± 0.26	—	—	—	—	—

[1] Average initial weight, 49 g.
[2] Dry weight basis.
[3] SD.

sources. The average weight gained per dietary protein level for each of the protein sources was similar, except in the groups fed the 30% protein level, where the soybean protein textured food showed an unexpected weight decrease. Weight gain reached a plateau with diets containing 16.7, 16.3 and 17.0% protein, for the soybean protein textured food, casein and dehydrated beef, respectively. Food intake was also very similar for all proteins within each protein level in the diet.

The table also shows values for total serum protein, albumin concentration, and the fresh weight of the liver and liver fat, with respect to protein level of intake for two of the protein sources. In both cases total serum protein increased as dietary protein level was raised in the diet. The increase was similar for both protein sources. Similar tendencies were found in albumin content, and the values found at each level of protein intake were similar, except at the lowest level, where the soybean protein textured food gave a lower value than casein and was statistically significant at the 5% level.

Fresh liver weight correlated with body weight for both protein sources. Liver fat decreased as protein in the diet increased. However, it remained essentially the same for protein levels above 16% in the diet.

Table 4 shows the PER and NPU of the protein isolate, fiber and soybean protein textured food as well as those of casein. The isolated protein had the lowest PER, followed by the protein fibers. The PER of the soybean protein textured food gave a value equivalent to 89% of the value of casein, which is similar to that found in the previous study. NPU results correlated with PER values, with the exception of the protein isolate.

TABLE 4

PER and NPU values of isolate, fiber and soybean protein textured food (12 rats/group)

Protein	Avg wt gain [1]	PER	NPU
	g		
Isolate	5 ± 10 [2]	0.33 ± 0.30	39.0 ± 9.4
Fiber	59 ± 14	1.90 ± 0.31	36.7 ± 6.5
Textured food	123 ± 26	3.01 ± 0.27	56.5 ± 8.6
Casein	114 ± 28	3.38 ± 0.32	61.1 ± 8.9

[1] Average initial weight, 44 g.
[2] SD.

The results obtained with the isolate were unexpected, since the process used to obtain the product consists of a simple extraction with an alkaline reagent, and there was no reason to believe that it could cause damage to the protein. Since it has been shown that heat treatment is beneficial for soybeans, destroying trypsin inhibitors, it was decided to repeat the previously presented study, using heat-treated products. The results are shown in table 5. It is interesting that heat treatment of the isolate and of the fiber caused a significant increase in weight gain as well as in PER. Food intake for the isolate doubled upon heat treatment. Heating of the soybean protein textured food did not increase PER but a significant increase took place in weight gain, with a higher intake of

TABLE 5

Effect of cooking on the protein quality of the isolate, fiber and soybean protein textured food (8 rats/group)

Proteins	Wt gain [1]	Avg food consumed	PER
	g	*g*	
Isolate	−5 [2]	159	—
Fiber	59 ± 22 [3]	309	1.88 ± 0.56
Soybean protein textured food	98 ± 20	326	3.30 ± 0.25
Casein	118 ± 21	365	3.53 ± 0.30
Isolate (heat-treated)	81 ± 13	340	2.44 ± 0.32
Fiber (heat-treated)	72 ± 11	320	2.23 ± 0.34
Soybean protein textured food (heat-treated)	138 ± 20	414	3.38 ± 0.19
Casein (heat-treated)	130 ± 22	379	3.38 ± 0.28

[1] Average initial weight, 42 g.
[2] Three animals out of eight died.
[3] SD.

food. Only weight gain was affected by heating of casein.

Soybean protein is known to be deficient in sulfur-containing amino acids, and chemical and physical treatments are applied in the conversion of soybean protein to a simulated food, which could change amino acid availability. It was, therefore, of interest to learn whether or not an improvement in the nutritive value of the protein of the soybean protein textured food could be obtained by supplementing it with methionine and lysine.

Table 6 shows the results of the study. Supplementing the soybean protein textured food with lysine has no effect on weight gain and PER. The addition of methionine caused only a slight increase in both PER and weight gain. When both amino acids were added, however, there was a significant increase in both parameters. Table 7 summarizes the results of similar studies, in which raw and cooked soybean protein textured food was supplemented with the same 2 amino acids. Examination of the corrected PER values indicated that cooking caused an increase in PER with and without amino acid supplementation. The soybean protein textured food has a lower PER than dehydrated beef, which gave also higher values than casein.

Dogs. The growth of the two groups of dogs is shown in figure 1. The growth of the dogs fed the soybean protein textured food was essentially the same as the growth of the animals fed the natural beef diet. The average total food intake, as well as the protein ingested, is also shown in the table. Food intake was higher for the animals fed the soybean protein textured food; however, the protein content of the diet was lower resulting in a lower protein intake as compared with that of the animals fed the dehydrated beef-containing diet. PER values were calculated from the weight gained and the protein consumed. These are essentially equal. Even though the animals were fed large amounts of the soybean protein textured food, no adverse physiological effects were observed.

The nitrogen balance data are presented in table 8. Higher nitrogen retention values appear to occur for the textured food at the higher levels of protein intake. Nitrogen balance was only slightly higher for the protein of dehydrated beef than for the soybean protein textured food at the lower levels of nitrogen intake. Negative

TABLE 6

Effect of methionine and lysine supplementation on the PER of soybean protein textured food (SPTF) (18 rats/group)

Treatment	Wt gain [1]	PER
	g	
Soybean protein textured food	114 ± 16 [2]	2.64 ± 0.21
SPTF + 0.25% L-lysine·HCl	120 ± 19	2.71 ± 0.23
SPTF + 0.30% DL-methionine	123 ± 20	2.81 ± 0.25
SPTF + 0.25% L-lysine·HCl + 0.30% DL-methionine	130 ± 24	2.95 ± 0.34

[1] Average initial weight, 49 g.
[2] SD.

TABLE 7

Effect of methionine and lysine supplementation to the soybean protein textured food (SPTF) (12 rats/group)

Treatment	Wt gain [1]	PER
	g	
Soybean protein textured food	123 ± 23 [2]	2.57 ± 0.19
(SPTF) (re-cooked)	131 ± 22	2.68 ± 0.22
SPTF + lysine + methionine	123 ± 24	2.76 ± 0.30
SPTF + lysine + methionine (re-cooked)	121 ± 17	2.97 ± 0.25
Casein	122 ± 21	2.77 ± 0.18
Dehydrated beef	149 ± 26	3.07 ± 0.28

[1] Average initial weight, 45 g.
[2] SD.

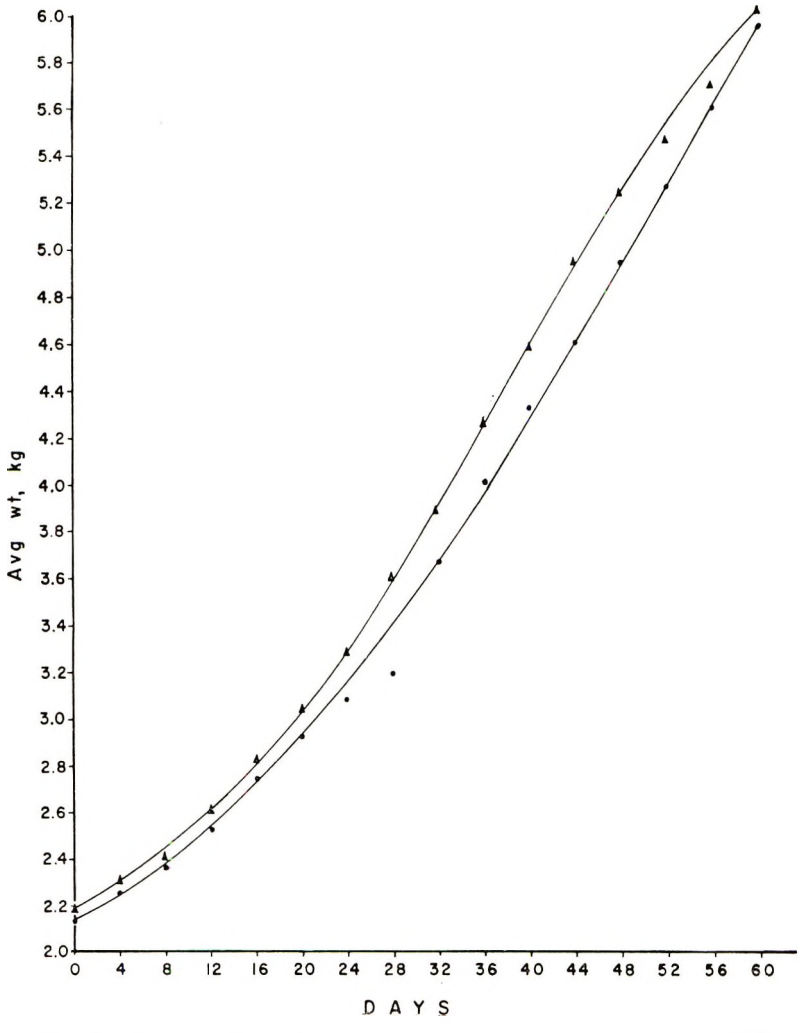

Fig. 1 Growth of young dogs fed soybean protein textured food and dehydrated beef; Average 6 dogs/group. ▲, dehydrated beef; ●, soybean protein textured food.

	SPTF	Dehydrated beef
Wt gain, g/day	62.7	63.8
Food consumed, g	6325	5835
Protein intake, g	1714	1921
PER	2.20	2.00

balances were obtained at intakes of nitrogen under 200 mg N/kg/day. Despite these differences, the results indicate that the nutritive value of the two protein sources is essentially the same. Weight gains were higher for dogs fed dehydrated beef. The animals started losing weight at nitrogen intakes below 100 mg/kg/day. True protein digestibility and biological value were 90.0 and 68.3%, respectively, for the soybean protein textured food, calculated at a nitrogen intake of 261 mg/kg body weight/day. For dehydrated beef, true protein digestibility was 87.0% and its biological value 67.4%, calculated at the 292-mg nitrogen intake level.

Children. Table 9 shows the nitrogen balance results in the children fed whole milk and the soybean protein textured food at an intake of 2 g of protein/kg/day. Nitrogen absorption and retention were essentially the same for both protein foods at this level of protein intake. The differences in nitrogen intake and retention between the 2 groups were not statistically significant, although they were so with respect to nitrogen absorbed when expressed on an absolute basis. Table 10 shows nitrogen balance results in the children fed decreasing levels of protein intake from the soybean protein textured food. Nitrogen retention and absorption decreased as nitrogen intake decreased. Regression lines between nitrogen intake (NI) and nitrogen retention (NR) and between nitrogen absorption (NA) and nitrogen retention (NR) were calculated from nitrogen intakes below 160 mg. The first equation was $NR = -59.8 + 0.433$ NI ($r = 0.64$) and the second, $NR = -44.7 + 0.534$ NA ($r = 0.61$). Nitrogen equilibrium was obtained when the children received approximately 138 mg of nitrogen from the soybean protein textured food, as compared with 97 mg from milk.[11]

DISCUSSION

From the initial studies with rats, the results indicate that the protein value of the soybean protein textured food is equivalent to about 92% of the nutritive value of casein and of dehydrated beef,

[11] Bressani, R., F. Viteri, D. Wilson, J. Alvarado and M. Béhar 1966 The protein value of several animal and vegetable proteins in children. Federation Proc., 25 (2, part 1): 299 (abstract).

TABLE 8

Nitrogen balance results in dogs

Protein	Nitrogen					Wt
	Intake	Fecal	Urine	Absorbed	Retained	
	mg/kg/day	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*kg*
SPTF [1]	630 ± 20 [2]	65 ± 16	228 ± 35	565 ± 25	337 ± 45	7.416
DB [3]	619 ± 28	111 ± 18	247 ± 27	508 ± 27	261 ± 26	8.084
SPTF	516 ± 42	63 ± 15	188 ± 22	453 ± 44	265 ± 51	8.408
DB	—	—	—	—	—	—
SPTF	419 ± 23	63 ± 11	158 ± 38	356 ± 26	198 ± 45	8.741
DB	469 ± 24	98 ± 27	211 ± 24	371 ± 30	160 ± 32	8.443
SPTF	261 ± 36	55 ± 13	138 ± 24	206 ± 32	68 ± 40	8.947
DB	292 ± 33	69 ± 21	149 ± 22	223 ± 24	74 ± 33	9.884
SPTF [4]	190	45	172	145	−27	9.462
DB	156 ± 16	66 ± 9	111 ± 29	90 ± 21	−21 ± 19	9.959
SPTF	99 ± 17	40 ± 7	102 ± 16	59 ± 15	−43 ± 25	9.074
DB	93 ± 5	43 ± 8	101 ± 25	50 ± 9	−51 ± 21	10.008
SPTF	48 ± 9	31 ± 7	70 ± 10	17 ± 7	−53 ± 14	9.065
DB	57 ± 15	38 ± 14	81 ± 16	19 ± 16	−62 ± 21	9.844
SPTF	0	23 ± 6	65 ± 14	—	—	8.596
DB	0	31 ± 11	66 ± 12	—	—	9.473

[1] SPTF = soybean protein textured food.
[2] SD.
[3] DB = dehydrated beef.
[4] Average of one dog for 2 four-day balance periods.

TABLE 9

Nitrogen balance of children fed milk and soybean protein textured food (SPTF) at 2 g protein/kg/day

Protein food	Nitrogen						
	Intake	Fecal	Urine	Absorbed	Retained	Absorption	Retention
	mg/kg/day	*mg/kg/day*		*mg/kg/day*	*mg/kg/day*	*% of intake*	
Milk	342	52	210	290	80	84.8	23.4
SPTF	312	46	183	266	82	85.2	26.6
t value (df 31)	1.43(ns) [1]			2.99(s) [2]			0.10(ns)

[1] Not significant.
[2] Significant.

TABLE 10

Nitrogen balance of children fed decreasing levels of protein from soybean protein textured food

Protein intake	Nitrogen						
	Intake	Fecal	Urine	Absorbed	Retained	Absorption	Retention
g/kg/day	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*% of intake*	
1.5	231 ± 14 [1]	42 ± 11	140 ± 11	190 ± 11	49 ± 10	81.8	21.2
1.0	156 ± 7	36 ± 14	111 ± 13	120 ± 15	9 ± 16	76.9	5.8
0.75 [2]	114 ± 10	32 ± 3	92 ± 13	82 ± 12	−10 ± 9	71.9	−8.8
0.50	78 ± 3	30 ± 7	75 ± 12	48 ± 7	−27 ± 16	61.5	−34.6
—	—	24 ± 6	61 ± 13	—	—	—	—

[1] SD.
[2] Average of 5 children only.

based on the PER values obtained at the 11.6, 11.7 and 12.2% protein diet, respectively. The soybean protein textured food gave essentially the same weight gain as casein and dehydrated beef at comparable protein levels. The palatability of the food was extremely good as judged from the amount of food consumed by the rats, varying between 347 to 441 g/rat in 28 days. The blood chemistry of the animals fed the soybean protein textured food was similar to the blood picture obtained with animals fed casein. Fresh liver weight correlated with body weight for both casein and the soybean protein textured food. High fat content of the liver in rats fed the low protein level is commonly found, and it concurs with results of investigators working with various protein sources at low levels of protein intake. It is known that low protein diets, in spite of good amino acid balance, cause accumulation of fat in the liver (7–9).

The NPU method of protein evaluation applied to the soybean protein textured food also indicated that it has a slightly lower nutritive value than casein and, on a relative basis, 94.4% of the casein value concurring with calculations based on PER. The low PER value for the protein isolate can be attributed to the presence, in the protein, of residual soybean growth inhibitors, or to other substances derived from the process of extraction and precipitation of the protein. From the results it appears that either the inhibitor or another substance is eliminated in the process of changing the isolate into fiber. The significant increase in both PER and NPU from the fiber to the final product is probably the result of egg albumin being added, eliminating in this manner at least part of the methionine deficiency inherent in soybean protein (10, 11). In general, PER correlated well with NPU for all products, except for the isolate. However, the discrepancy can be explained on the basis of the duration of the test for each assay. NPU assays run for 10 days (4) while PER assays take 28 days, permitting any physiologically adverse substance to accumulate and act against the performance of the animal. The animals consuming the isolate gained weight during the first 2 weeks of the PER assay, but lost weight during the last two, which resulted in lower growth and PER values.

The results obtained when the materials were cooked indicate that this process eliminated the growth-inhibiting substances present in the isolate. Very little effect was obtained when the fiber or the final product were heated, although there was a definite tendency to consume more food. It is well known that soybean flour must be properly heat-treated to obtain maximal nutritional value, although excess heat can decrease its protein quality (10, 12, 13). Improvement in the protein quality resulting from controlled heat treatment is due to the destruction of soybean trypsin inhibitors (14, 15), and to probable modifications of the protein, permitting a more complete digestibility and utilization of the sulfur amino acids, which are limiting in soybean protein (13, 15, 16). Recently, Longenecker et al. (17) reported that soybean protein isolate may contain good quality protein, but often requires mild heat treatment to bring out the maximal protein value.

From the results of amino acid supplementation, the soybean protein textured food appears to be mildly deficient in both

lysine and methionine, since the addition of both acids together increased PER values particularly when the food was further heat-treated. The effect was not as marked when the material was unheated, suggesting that the treatment applied, although mild, probably caused some decrease in the availability of both amino acids. It is also probable that even the final product still contained some inhibitor which did not allow for the supplements to express their beneficial effects on increasing protein quality. The response obtained with young growing dogs fed the soybean protein textured food was similar to that obtained with dehydrated beef, indicating again its excellent protein quality. No adverse physiological effects were noted during the entire experimental period. Assuming that the final food still contained physiologically adverse substances, these did not show their effect on the dogs, probably because the food was heated before consumption, thus eliminating the possible factors. The nitrogen balance results obtained with dogs indicate again the high protein quality of the soybean protein textured food studied.

With respect to the studies with children, the soybean protein textured food was readily accepted by all and there were no adverse effects at any time during the experiment.

Using the endogenous fecal and urinary nitrogen excretion values, and the nitrogen balance values at the 1 g of protein level of intake, the true protein digestibility and the biological value of the protein in the soybean protein textured food was calculated according to the formula of Mitchell (4, 18). These values are 92.3 and 65.3%, respectively. At an intake of 0.75 g of protein per kg body weight, true protein digestibility was found to be 93.0%, and the biological value, 70.8%.

Similar calculation from data obtained on children of ages similar to the ages of the subjects used in this study, and fed milk protein, indicated that the true protein digestibility and biological value are 92.0 and 80.6%.[12]

It can be concluded then that the protein quality of the soybean protein textured food is high (about 80% of the protein quality of milk), with adequate digestibility; it is readily acceptable and free of adverse physiological effects.

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Effects of Thyroid Hormone and Phosphorus Loading on Renal Calcification and Mineral Metabolism of the Rat

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ABSTRACT Seventy-two young male albino rats were used in 2 experiments designed to study effects of chemical blockage of thyroid secretion and of supplemental thyroxine administration on renal calcification and other criteria of mineral metabolism as influenced by high phosphate intake. Calcium content of kidney mitochondria and whole kidney tissue was increased by high phosphate diets and was inversely affected by level of thyroid activity; phosphorus content of whole kidney tissue, but not of mitochondria, was similarly affected. Histological and chemical studies of kidneys revealed a parallelism between calcification and PAS-stainable material. Percentage retention of dietary calcium and magnesium was markedly depressed in both hypo- and hyperthyroid animals. High phosphorus diets decreased the percentage of calcium and magnesium absorption and their excretion in the urine, and hence had little effect on balance. The effects of high phosphate diets on kidney calcification and urinary mineral excretion resemble those of parathyroid hormone and may be a result of induced parathyroid activity. The effect of thyroid hormone on reducing kidney calcification may be related to its effect on mucopolysaccharide metabolism.

Soft tissue calcification has been reported to result from high phosphorus intakes (1–3). Robbins et al. (4) found that diets containing high concentrations of phosphorus tend to increase the formation of urinary calculi of a magnesium-phosphate type in lambs. Although there was little change in serum phosphate, increases in the urinary excretion of both calcium and phosphorus were pronounced.

MacKay and Oliver (1) identified morphological changes in the renal tubules of rats fed a high phosphorus diet. They found that the entire outer stripe of the outer zone of the medulla of the kidney was transformed into a zone of distorted structures and that there was an increase in interstitial connective tissue.

Renal calcification, normally accompanying magnesium deficiency, has been shown to be inhibited by supplemental thyroxine administration (5–7). The action of thyroid hormone in opposing the deposition of calcium compounds in renal tubules and in general mineral metabolism is still unclear.

In the work to be reported, renal tissue was observed histologically and chemically in rats soon after they began consuming diets normal or high in phosphorus and with or without thyroxine or propylthiouracil. Renal mineral composition and mineral balance were determined after 3 weeks of dietary treatment. The data verify some previous findings and help to clarify the role of thyroid hormone in mineral metabolism.

METHODS

Male rats of the Sprague-Dawley strain, weighing approximately 125 g, were housed in stainless steel metabolism units at a controlled temperature of 23 to 25°. Basal and high phosphate diets, containing 0.51% and 0.90% phosphate, respectively, were prepared as shown in table 1. Each diet was fed unsupplemented, or after the addition of 10 ppm L-thyroxine (T_4) as the sodium salt, or 0.15% propylthiouracil [2] (PTU), thus providing animals with 3 levels of thyroid activity at each of 2 levels of dietary phosphate. During a preliminary 2-week period all rats received the basal level of phosphorus; one-third of them received PTU-supplementation to accomplish

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TABLE 1

Composition of diets

	% of diet
Casein [1]	15.0
DL-Methionine	0.4
Cellulose [2]	3.0
Corn oil	10.0
Lactose [3]	25.0
Glucose [4]	38.2
Cod liver oil [5]	0.5
Vitamin mixture [6]	5.0
Mineral mixture [7]	2.9
Total	100.0
$NaH_2PO_4 \cdot H_2O$ [8]	

[1] Vitamin-Free Test Casein, Whitson Products, Borden Company, New York.
[2] Solka Floc, Brown Company, Boston.
[3] Milk sugar Merck, Merck and Company, Inc., Chemical Division, Rahway, New Jersey. Lot no. E-32033.
[4] Cerelose, Corn Products Company, New York.
[5] Parke-Davis standardized cod liver oil, 2000 USP units of vitamin A and 250 USP units of vitamin D/gram.
[6] Contained in mg/kg of mixture: thiamine·HCl, 200; riboflavin, 120; pyridoxine·HCl, 80; Ca pantothenate, 320; biotin, 4; niacin, 500; folic acid, 10; vitamin B_{12}-mannitol (B_{12} present at 0.1%), 100; menadione, 6.6; choline chloride, 30,000; glucose to make 1000 g.
[7] Contained in percent of mixture: NaCl, 12.73; K_2CO_3, 17.30; $FeSO_4 \cdot 7H_2O$, 1.27; $MnSO_4 \cdot H_2O$, 0.41; $CuSO_4 \cdot 5H_2O$, 0.20; KI, 0.013; $ZnCO_3$, 0.077; $CaHPO_4$, 47.0; $MgCO_3 \cdot Mg(OH)_2 \cdot nH_2O$, 5.0; and $NaH_2PO_4 \cdot H_2O$, 16.0.
[8] Added at 2.22% of diet in high phosphorus diet at the expense of glucose.

blockage of thyroxine production. This period allowed depletion of endogenous stores of thyroid hormone in PTU-treated animals. All diets contained 0.38% calcium and 380 ppm magnesium.

Feed intake was initially equalized among groups and was governed by the group having the lowest voluntary intake. During the third week, the PTU-treated animals consumed only 7 to 8 g of diet per day; therefore, normal and hyperthyroid animals were allowed to consume 10 to 11 g in order to prevent excessive weight loss in the latter group. Demineralized water was available at all times.

Two phases of the experiment were conducted concurrently. Seventy-two animals were used, 48 in a 6-day time study and 24 in a 21-day metabolic study. Animals were housed two per cage in the time study, and 6 groups of 8 animals were formed by random selection. Two animals from each group were killed after 1, 2, 4 and 6 days on dietary treatment. Six groups of 4 animals selected at random, housed individually, were used in the 3-week study. The experimental diets were fed to these animals for 21 days, and excreta were collected during the concluding 7 days. These 6 groups were killed by exsanguination at 21 days, and kidney samples were taken for analysis.

One of the pair of kidneys from each animal was immediately sectioned longitudinally and one-half placed in 10% buffered formalin fixative, while the other half was frozen for chemical analysis. The contralateral kidney was homogenized fresh, and the mitochondria were separated according to the method of Vasington and Murphy (8), as modified by Christie et al. (9). This subcellular fraction was then ashed and analyzed for mineral content.

Protein was determined on aliquots of kidney homogenate in the manner described by Lowery et al. (10).

Blood samples were drawn from the abdominal aorta, and chemical analyses were performed on the serum. Samples of diet, kidney and kidney mitochondria were wet-ashed with nitric acid followed by hydrogen peroxide. Analyses for calcium and magnesium were performed by standard procedures using the Perkin-Elmer Model 303 atomic absorption spectrophotometer. Phosphorus was determined by the method of Fiske and Subbarow (11) as modified by Clark (12). Graphol [3] was substituted for Elon in the reducing reagent, and samples were read at 660 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. Glycoprotein in serum was determined as protein-bound hexose in the manner described by Winzler (13).

Histological study was made of kidney sections which were fixed, mounted in paraffin blocks and sectioned at a thickness of 7 to 10 μ. Serial sections were stained by standard procedures with either hematoxylin-eosin to show general morphology or Periodic Acid-Schiff stain (PAS) for evaluation of mucopolysaccharide content.

The data were analyzed statistically by analysis of variance procedures (14) for the 2 × 3 factorial design, and the levels of significance are indicated in the results. Additional non-orthogonal, single degree of

[3] Mono - methyl - para - aminophenol - sulfate, photographic grade, ASA spec. pH 4.125. Hunt and Company, Palisades Park, New Jersey.

freedom comparisons were made to assess the significance of the effect of thyroxine on early kidney mitochondrial calcium concentration.

RESULTS

The changes shown by histological and chemical examination of kidney tissue removed from animals 1, 2, 4 and 6 days after commencing experimental treatment could not be correlated with time, therefore data for these 4 periods were combined and will be disussed as "one-week combined" data.

Data from chemical analysis of ashed kidney tissue, summarized in table 2, show increased calcium and phosphorus due to elimination of thyroid function ($P < 0.005$). Kidney mineral content was not influenced by supplemental thyroxine or increasing dietary phosphorus during the first week of treatment. After 3 weeks of treatment, kidneys from animals devoid of endogenous thyroxine due to PTU treatment contained the greatest concentration of calcium ($P < 0.005$), while supplemental thyroxine in the diet depressed such calcium deposition ($P < 0.005$) and high phosphorus intake enhanced it ($P < 0.05$). The concentration of phosphorus was reduced by supplemental thyroxine ($P < 0.025$) and was increased by phosphorus supplementation ($P < 0.025$). Supplemental phosphorus increased magnesium concentration except in the presence of supplemental thyroxine ($P < 0.025$).

Table 3 shows that the mineral content of mitochondria isolated from kidneys of animals receiving the high phosphorus treatments contained more calcium than those from animals receiving the basal level of phosphorus ($P < 0.05$). Phosphorus and magnesium content was unchanged by increasing the dietary phosphorus. Level of thyroid activity in the animals did not affect phosphorus or magnesium content of mitochondria, but an inverse relationship existed between mitochondrial calcium and thyroid activity.

The results of serum analysis are shown in table 4. Animals devoid of endogenous thyroxine due to PTU treatment showed decreased serum phosphorus ($P < 0.005$), while T_4-treated animals exhibited elevated

TABLE 2

Effects of thyroxine and dietary phosphorus on mineral content of kidneys during the first week and at 3 weeks

Dietary treatment	Calcium		Phosphorus		Magnesium	
	Week 1	Week 3	Week 1	Week 3	Week 1	Week 3
			mg/g dry tissue			
Basal + PTU [1]	8.7	12.2	17.4	15.4	1.18	1.16
Basal	3.1	7.0	14.6	14.1	1.11	1.06
Basal + T_4 [2]	3.3	1.6	14.0	11.9	1.15	1.04
High phosphate + PTU	11.2	16.0	17.3	18.7	1.28	1.34
High phosphate	4.0	15.8	15.4	18.9	1.14	1.41
High phosphate + T_4	2.3	2.4	14.1	13.1	1.15	1.03

[1] PTU indicates propylthiouracil.
[2] T_4 indicates L-thyroxine.

TABLE 3

Effect of thyroxine and dietary phosphorus on mineral content of kidney mitochondria (one-week combined data)

Diets	Calcium	Phosphorus	Magnesium
		μg/mg N	
Basal + PTU [1]	2.23	18.2	0.59
Basal	1.97	18.9	0.68
Basal + T_4 [2]	1.43	16.8	0.61
High phosphate + PTU	3.10	16.2	0.63
High phosphate	2.69	16.8	0.58
High phosphate + T_4	2.06	17.0	0.59

[1] PTU indicates propylthiouracil.
[2] T_4 indicates L-thyroxine.

TABLE 4

Effect of thyroxine and dietary phosphorus on mineral composition and glycoprotein content of blood serum (one-week combined data)

Dietary treatment	Calcium	Phosphorus	Magnesium	Glycoprotein
		mg/100 ml		
Basal + PTU [1]	10.2	7.2	2.09	120.5
Basal	10.2	9.4	1.90	101.7
Basal + T_4 [2]	9.8	11.2	1.95	92.4
High phosphate + PTU	10.5	7.9	1.95	124.4
High phosphate	10.3	8.5	1.88	102.8
High phosphate + T_4	10.1	10.2	2.06	97.2

[1] PTU indicates propylthiouracil.
[2] T_4 indicates L-thyroxine.

levels ($P < 0.005$). The magnitude of depression due to PTU treatment was greater in animals receiving the basal phosphorus diets ($P < 0.05$). Contrary to expectation, increasing the dietary phosphorus had no significant effect on serum phosphorus early in the treatment period. Hypothyroidism caused an elevation of serum glycoprotein ($P < 0.005$), while a trend toward depression of this component in hyperthyroid animals is shown.

Histological examination of kidneys taken during the first week showed changes only in renal tubular cells in the area of the cortico-medullary junction. Hematoxylin-eosin stained sections displayed dilated tubules with shrunken cells lining the lumen in the inner cortex and outer stripe of the medulla in kidneys from animals receiving PTU or high phosphorus treatment only. By 21 days it was evident that there was loss of integrity of the tubular epithelium in kidneys of animals receiving PTU or high phosphorus diets, and cells of the epithelium had begun to slough into the lumen of some tubules.

Coincident with these changes, there was an accumulation of PAS-positive material, interpreted as mucopolysaccharide, and deposition of calcium compounds in the cortico-medullary junction region. Figure 1 is a scattergraph of the results of light-microscopic evaluation of PAS-positive material in kidney compared with calcium found chemically in the other half of corresponding kidneys. The graph illustrates that the buildup of calcium and accumulation of PAS-positive material bear a direct relation. No information was gained to indicate that PAS-positive material accumulates before deposition of calcium or vice versa.

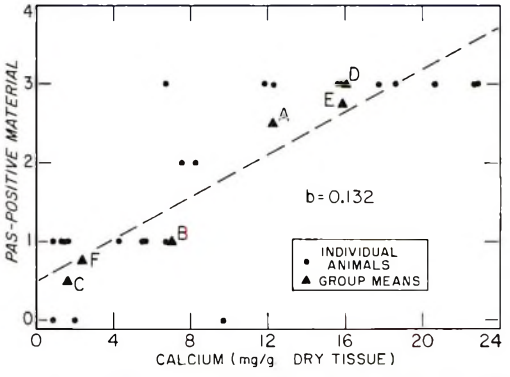

Fig. 1 Effect of thyroxine (T_4) and dietary phosphorus on the relationship of calcium to mucopolysaccharide material found histochemically in kidney sections at 21 days. Group *A*, basal + propylthiouracil (PTU); *B*, basal; *C*, basal + L-thyroxine (T_4); *D*, high phosphate + PTU; *E*, high phosphate; and *F*, high phosphate + T_4.

Although feed intake was initially equalized among groups at 10 g daily, the PTU-treated animals failed to continue to consume this amount and averaged an intake of about 8 g daily. Differences in gain associated with phosphorus content of the diet were not large, but PTU-treated, normal and thyroxine-treated animals averaged 0.49, 2.01 and − 0.41 g daily over the 3-week period and during the week of collection averaged 0.7, 1.6 and − 1.7 g daily.

The apparent absorption and urinary excretion patterns of the animals during the concluding week of the 3-week study are shown in table 5 in which the data are expressed as a percentage of the intake. Absorption of calcium, phosphorus and magnesium was depressed in both hypo-

TABLE 5

Effects of dietary treatment on weight gain and on mineral balance

Dietary treatment	Feed intake	Final wt	Calcium		Phosphorus		Magnesium	
			Abs.	Urine	Abs.	Urine	Abs.	Urine
	g/day	*g*	*% of intake*		*% of intake*		*% of intake*	
Basal + PTU [1]	6.9	113	34	18	72	52	61	46
Basal	10.4	186	68	6	85	27	84	57
Basal + T_4 [2]	10.5	130	29	8	75	32	76	68
High phosphate + PTU	8.0	126	30	8	80	66	46	33
High phosphate	10.5	180	56	4	89	76	81	54
High phosphate + T_4	10.5	129	23	4	82	80	73	62

[1] PTU indicates propylthiouracil.
[2] T_4 indicates L-thyroxine.

and hyperthyroid animals ($P < 0.05$), with the exception that the depression of magnesium absorption in T_4-treated animals failed to reach significance at the 5% level. Phosphorus supplementation depressed magnesium absorption ($P < 0.05$), elevated phosphorus absorption ($P < 0.005$), and tended to depress calcium absorption.

Urinary excretion of calcium was reduced in animals receiving supplementary phosphorus ($P < 0.005$) and was increased in those animals receiving PTU ($P < 0.005$). Propylthiouracil enhanced urinary phosphorus excretion ($P < 0.005$) of animals receiving the basal phosphorus level, an effect not seen at high phosphorus intake due to the marked increase in urinary phosphorus in this latter treatment. Urinary magnesium was reduced in animals receiving high phosphorus intakes or PTU treatment ($P < 0.005$), but was elevated in hyperthyroid animals ($P < 0.005$).

DISCUSSION

Kidney mitochondria obtained during the first week of the experiment showed a higher content of calcium as a result of increased phosphorus intake, while there was little change in mitochondrial content of phosphorus or magnesium. Mitochondrial calcium content was inversely related to the thyroid activity of the animals. These data are indicative of early abnormal concentration of calcium by kidney mitochondria under the influence of dietary mineral and hormone imbalance, although concomitant increases in phosphorus and magnesium do not occur.

In addition, alteration of the thyroxine-dependent respiration rate may affect mineral transport in mitochondria as suggested by the lower calcium content as available thyroxine increased.

Analysis of kidneys showed that blockage of thyroid function with PTU resulted in an elevated deposition of calcium and phosphorus, but there was no effect on the renal content of magnesium. Contrary to results of mitochondrial analysis, alteration of renal mineral deposition within the first week did not result from high phosphorus intakes. By 21 days, kidneys from animals devoid of endogenous thyroxine contained significantly more calcium, while supplemental thyroxine prevented this deposition of calcium as well as that of both phosphorus and magnesium. Thus the data for mitochondria and whole kidney agree in part. High phosphorus intakes for 3 weeks resulted in increased renal content of all three of these minerals, an effect not yet evident at one week of dietary treatment. These results may be further evidence that calcification involves some early changes in subcellular mineral transport and that such changes may be related to thyroxine-dependent tissue metabolism.

Thyroid blockage depressed serum phosphorus as opposed to elevated levels under the influence of supplemental thyroxine. These data may further confirm that the metabolic rate is an important factor in the regulation of circulating and tissue mineral levels. Findings by Fourman (15) that phosphorus-loading did not increase serum phosphorus were substantiated.

Lower serum glycoprotein levels in animals receiving thyroxine paralleled decreased histologically detectable mucopolysaccharide material in the kidney and may indicate that synthesis or liberation of previously synthesized mucopolysaccharide

components is dependent on metabolic activity. It may be that the potentiating effect of thyroxine on renal calcification is due to a decrease in mucopolysaccharide material acting as a matrix for calcium deposition.

Several groups of workers (16–18) believe that mucopolysaccharide materials lodge in kidney tubules and act as a matrix for calcification. The morphological changes in kidney accompanying calcification in this experiment were consistent with early reports by MacKay and Oliver (1). An attempt to identify possible mucopolysaccharide material which would coincide with calcium deposition early in the calcification process showed a direct proportionality between the amount of calcium and PAS-positive material interpreted as mucopolysaccharide. It seems likely that materials which react positively to histochemical tests for mucopolysaccharides may be secreted into kidney tubules by lumen epithelial cells in the cortico-medullary junction region of the kidney during the calcification process. It could not be determined whether mucopolysaccharide had accumulated before or after calcium deposition, although Boyce and King (19) believe the organic matrix to be prerequisite to calculus formation.

Urinary excretion of calcium was increased in PTU-treated animals, while the excretion of magnesium was depressed when excretion was expressed as percentage of intake. Supplemental thyroxine stimulated urinary excretion of magnesium but not of calcium. Increased urinary phosphorus excretion was noted in hypothyroid animals receiving the basal diet, but this result was masked by high phosphorus intakes, under the influence of which phosphorus excretion was much higher regardless of thyroid status. Contrary to the results of Fourman (15), large intakes of phosphorus sharply depressed urinary calcium, and there was a smaller depression of urinary magnesium.

The depression of urinary calcium and magnesium by high phosphorus intake as well as the increased mineral deposition in kidney tissue resembles closely the effect of parathyroid hormone and, therefore, may be a result of alteration of renal tubular reabsorption of calcium and magnesium, possibly mediated through hormone action.

Decreased mineral absorption resulting from supplemental thyroxine is in agreement with the work of Forbes (7). Because PTU treatment resulted in an equally large depression in mineral absorption, the effect of thyroxine on intestinal transport probably has little relation to its amelioration of renal calcification. Since the absorption of calcium and magnesium was depressed while that of phosphorus was elevated by high phosphorus intakes, the increased load of phosphorus presented to the kidney tubule may account for damage in the cortico-medullary junction region leading to calcification.

The major effects on mineral absorption and excretion seen in this study may reflect either direct effects on mineral transport as a result of the level of thyroid activity or indirect effects related to mineral requirements and supply. For example, the lower balance of calcium in the T_4-treated animals may be a result of the general catabolic effect of thyroxine which, under the conditions of equal feed intake imposed, inhibited deposition of all nutrients as shown by the weight-gain data. That restriction of feed intake need not influence the percentage of mineral retention may be seen in the publication by Forbes (20) in which young rats gained 0.6 or 3.0 g daily as a result of differential feed intake in response to zinc supply. Yet, both groups of animals retained almost identical percentages of the dietary calcium, phosphorus and magnesium intake. It is believed that the effects just described are indicative that dietary mineral imbalance may result in physiologically significant increases in parathyroid hormone secretion which alter mineral excretion and induce the deposition of mineral in renal tissue. The reason for the ameliorating effect of supplemental thyroxine is still unclear, but may be related to greatly decreased histochemically detectable mucopolysaccharide in the kidneys of animals receiving supplemental thyroxine. Decreased serum glycoprotein may be an additional indication that there is regulation of the rate of synthesis of such complex molecules by thyroid hormone.

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Utilization of Amino Acids from Protein by Weanling Pigs [1,2]

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ABSTRACT The utilization by weanling pigs of amino acids from 3 sources was studied during a 2-week period. The sources were milk protein (a 5:1 mixture of casein and lactalbumin), wheat gluten and pure amino acids. Intakes of total nitrogen and essential amino acids were equalized by supplementing the proteins with pure amino acids to the FAO provisional pattern. Energy requirements were met by supplying nitrogen-free diet ad libitum. When all the nitrogen was supplied as amino acids less nitrogen was retained and more fat deposited than when part of the nitrogen was supplied as casein-lactalbumin. These effects may be related to a lack of glutamine and asparagine in the amino acid diet, coupled with a high requirement for ammonia to neutralize amino acid hydrochlorides in this diet. Poor utilization of wheat gluten was shown by nitrogen balance, weight gains, and other measurements. Differences in liver weights were due to differences in the non-fat, non-protein fraction of liver.

While it is well-known that proteins contain different amounts of the essential amino acids, there remains a question of how much of the amino acid content is available to man and animals. To help answer this question, 10 Experiment Stations cooperated in Northeastern Regional Project NE-52 to compare the utilization of amino acids from 3 sources: pure amino acids, milk protein (casein-lactalbumin mixture), and wheat gluten. Human subjects, rats, pigs, and protozoa were fed 3 diets, each of which supplied the FAO provisional pattern of amino acids but from the different sources. The FAO provisional pattern was used for all species and all physiological conditions (growth, maintenance, and stress) because a standardized pattern based on the needs of man was preferred in the initial studies.

The 3 sources of essential amino acids were as follows: (a) Pure amino acids in the FAO provisional pattern;[3,4] (b) a 5:1 casein-lactalbumin mixture [5] supplemented with pure amino acids to supply the FAO provisional pattern; and (c) wheat gluten [6] supplemented with pure amino acids to supply the FAO provisional pattern.

The proteins supplied some nonessential amino acids. A mixture of L-amino acids (alanine, arginine, aspartic acid, glutamic acid, histidine, proline, and serine) and glycine in the proportions found in milk (1) was added to each diet to bring the nitrogen content to the FAO provisional pattern. The sources of amino acids in the animal diets are listed in table 1. The amino acids in the human diets were formulated in the same manner except that the amounts of nonessential amino acid mixture were reduced to compensate for the small quantity of nitrogen supplied by the low protein foods in the USDA diet (2).

To assure comparability of results between stations, the amino acid and protein mixtures used by each station were drawn from the same lots. Stations working with a given species used the same diet formula, source of animals, experimental design, and methods of chemical analyses.

This paper presents responses of weanling pigs to equal amounts of essential amino acids and nitrogen derived from the 3 sources described above.

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[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station.

[3] As specified in Food and Agricultural Organization Bulletin 16 (1957), and with 144 mg DL-methionine and 126 mg L-cystine/g of nitrogen; other amino acids in the L-form.

[4] The amino acid mixtures and the casein-lactalbumin mixture were prepared by General Biochemicals, Chagrin Falls, Ohio.

[5] See footnote 4.

[6] The wheat gluten was from a single lot of General Mills Pro-80 Vital Wheat Gluten, prepared by a variation of the Martin process and dried in high vacuum at mild temperatures.

TABLE 1

Amino acid sources

	FAO	Amino acid diet	Casein-lactalbumin diet		Wheat gluten diet	
		Amino acids [1]	Protein [2]	Amino acids	Protein [2]	Amino acids
	mg/g dietary nitrogen		*mg/g dietary nitrogen*		*mg/g dietary nitrogen*	
Alanine	—	329	119	197	80	182
Arginine	—	349 [3]	115	209 [3]	112	193 [3]
Aspartic acid	—	697	262	417	96	387
Cystine	126	126	29	97	63	63
Glutamic acid	—	2242	722	1341	1163	1243
Glycine	—	189	60	113	112	105
Histidine	—	269 [3]	87	161 [3]	72	149 [3]
Isoleucine	270	270	187	83	108	162
Leucine	306	306	306	0	217	89
Lysine	270	270 [3]	257	13 [3]	53	217 [3]
Methionine	144	144	85	59	47	97
Phenylalanine	180	180	171	9	180	0
Proline	—	1066	328	637	514	591
Serine	—	563	187	337	148	312
Threonine	180	180	147	33	83	97
Tryptophan	90	90	52	38	30	60
Tyrosine	180	180	166	14	103	77
Valine	270	270	223	47	116	154
Ammonia	—	0	52	0	83	0
Nitrogen content	1000	1000	498	502	448	552

[1] NE-52 amino acid pattern.
[2] Cystine and tryptophan in the proteins were determined microbiologically by Prof. Pela Braucher at the University of Maryland. Other amino acids in the proteins were determined by ion exchange chromatography of acid hydrolysates by Prof. H. H. Williams at Cornell University.
[3] Added as an equivalent amount of the monohydrochloride.

PROCEDURES

Three identical experiments were run with 6 Yorkshire male littermate pigs in each. The pigs were weaned at 5 weeks of age, placed in metabolism cages, fed a purified pre-experimental diet (table 2) for one week, and one of the three test diets (table 2) for an additional 2 weeks. To prevent spillage losses and to minimize palatability and osmotic problems (3) these diets were fed twice daily in agar gel form. At both the 9 AM and 3 PM feedings, the agar gel diet was completely consumed within 5 minutes. To assure that the energy requirements were met for all sizes of pigs, additional dry, nitrogen-free diet (table 2) was supplied ad libitum throughout the study. Water, illumination, and room temperature of 19 to 25° were also provided 24 hours a day.

To maintain the rapidly growing pigs in approximately the same degree of protein deficiency throughout the experiment, the amount of nitrogen-containing diet was increased daily. The formula used was developed from the NRC nutrient requirements of swine (4). The practical nitrogen requirements given in that publication for pigs weighing 4.5, 11.3 and 22.7 kg (10, 25 and 50 pounds) can be expressed by the formula: grams nitrogen = 5.3 (kg body weight)$^{0.63}$. For these studies we reduced the coefficient from 5.3 to 1.7 to make nitrogen the limiting factor, and each day calculated the nitrogen to be fed from the average body weight of the 6 littermate pigs. Thus the nitrogen intakes were adjusted for differences in average size of the pigs in different litters and of different ages, but on any one day all pigs in an experiment were fed the same amount of nitrogen, despite individual differences in body size.

In all 3 experiments, 2 pigs each were fed the amino acid, casein-lactalbumin, or wheat gluten diet for 14 days. From days 8 to 13 inclusive, urine and feces were collected and frozen daily. Composite samples for the 6-day period were prepared and stored frozen until analyzed. Each urine collection bottle contained 10 ml 10% sulfuric acid.

At the end of each experiment, all pigs were killed at about 9 AM. They were stunned electrically (110 v AC ear-to-ear

TABLE 2
Composition of diets

	Pre-experimental [1]	Test diet components			
		Amino acid [1]	Casein-lactalbumin [1]	Wheat gluten [1]	Nitrogen-free supplement [2]
	%	%	%	%	%
Casein	15.6	—	—	—	—
Casein-lactalbumin [3]	—	—	10.8	—	—
Wheat gluten [4]	—	—	—	10.2	—
Amino acids [5]	6.7	23.9	11.7	13.0	—
Cornstarch	33.0	32.2	32.9	32.6	—
Sucrose	33.0	32.2	32.9	32.6	87.3
Corn oil	5.0	5.0	5.0	5.0	5.0
Cellulose [6]	—	—	—	—	2.0
Agar [7]	1.0	1.0	1.0	1.0	—
Mineral mix [8]	3.5	3.5	3.5	3.5	3.5
Vitamin mix [9]	2.2	2.2	2.2	2.2	2.2
Nitrogen, % dry matter	3.0	3.0	3.0	3.0	—
Energy, kcal/g dry matter [10]	—	4.232	4.307	4.371	3.999
Water added, g/g dry matter [11]	0.5	0.5	0.5	0.5	—

[1] Intakes restricted (see text).
[2] Available ad libitum to all pigs in all periods.
[3] A 5:1 mixture of casein and lactalbumin to simulate milk protein.
[4] General Mills Pro-80 Vital Wheat Gluten prepared by a variation of the Martin process and dried in high vacuum at mild temperatures.
[5] L-Amino acids (except DL-methionine) as specified in table 1. In the pre-experimental diet approximately three-fourths of the nitrogen was supplied as casein (supplemented with DL-methionine) and one-fourth as amino acids in the proportions specified for the amino acid test diet.
[6] Non-nutritive bulk, Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[7] Oxoid Ionagar no. 2, Calab Laboratories, Chicago Heights, Illinois; nitrogen content of this sample was 0.04%.
[8] Composition: (%) $CaHPO_4$, 57.50; KH_2PO_4, 7.50; NaCl, 16.60; K_2CO_3, 12.00; $MgCO_3$, 4.00; $FeSO_4 \cdot 7H_2O$, 1.50; $MnSO_4 \cdot H_2O$, 0.36; $CoCl_2 \cdot 6H_2O$, 0.12; $CuSO_4$, 0.10; NaF, 0.02; $ZnCO_3$, 0.30; and KI, 0.001.
[9] Contained (in %) vitamin A conc (200,000 IU/g), 0.45; vitamin D conc (400,000 IU/g), 0.025; α-tocopherol, 0.50; ascorbic acid, 4.50; inositol, 0.50; choline chloride, 7.50; menadione, 0.225; p-aminobenzoic acid, 0.50; niacin, 0.45; riboflavin, 0.10; pyridoxine·HCl, 0.10; thiamine·HCl, 0.10; Ca pantothenate, 0.30; biotin, 0.0020; folic acid, 0.0090; and vitamin B_{12}, 0.000135; Vitamin Diet Fortification Mixture, obtained from Nutritional Biochemicals Corporation.
[10] Calculated from gross energy values of the dietary components, except cellulose, agar, and minerals excluded. Bomb calorimetry measurements of the proteins and amino acids were made by Dr. George Barron at the Pennsylvania State University.
[11] The water, corn oil, and agar were heated to boiling, cooled to 60–65°, and added to the other premixed ingredients. All diets were stored in air-tight plastic containers at 3° and used within a few days.

for 20 seconds), suspended by the hind legs, and bled from the neck. The following organs were removed, weighed, and frozen for subsequent analyses: adrenals, heart, kidneys, liver, pancreas, spleen, and testes. Other viscera were discarded. The eviscerated carcass was allowed to hang in a refrigerator for 18 to 36 hours; the carcass was then bisected and length measurements were made of the vertebral column. The eviscerated carcasses, including the skin, head, and feet, were dried to constant weight in a forced-air oven (101°), and ground in a meat grinder and Waring blender; samples were frozen for analysis.

Moisture, ether extract, and nitrogen in livers, carcasses, and excreta were determined by the AOAC methods (5). Urea in urine was determined by Pellerin's adaptation (6) of the method of Coulombe and Favreau (7). Ammonia in urine was determined by microdiffusion, using Öbrink modified Conway units (8). Total creatinine (preformed creatinine plus creatine) and preformed creatinine in urine were determined by the method of Clark and Thompson (9), but with a 20:1 molar ratio of sodium hydroxide to picric acid. Nucleic acids in liver were determined by the method of Wannemacher et al. (10).

Statistical analyses were made by the analysis of variance with the variance partitioned among the dietary treatments, experiments (litters), and body weight classification (above or below the median weight for each experiment).

RESULTS

Average physical measurements of the live pigs and organs are given in table 3.

TABLE 3

Physical measurements [1]

	Dietary group		
	Amino acid	Casein-lactalbumin	Wheat gluten
Initial body wt,[2] kg	7.26 [a]	7.70 [a]	7.54 [a]
Final body wt, [2] kg	10.65 [a]	10.83 [a]	10.17 [a]
Wt gain,[2] g/day	242 [a]	223 [b]	188 [c]
Length of vertebral column,[3] mm	336 [a]	349 [a]	331 [a]
Organ wts, g			
Adrenals	0.85 [a]	1.01 [a]	0.87 [a]
Heart	47.9 [a]	52.0 [a]	48.4 [a]
Kidneys	48.4 [a]	48.3 [a]	49.3 [a]
Liver	284 [a]	273 [a]	245 [a]
Pancreas	11.6 [a]	11.6 [a]	11.9 [a]
Spleen	17.2 [a]	18.2 [a]	17.2 [a]
Testes	9.6 [a]	10.3 [a]	10.0 [a]
	Ratios to initial body weights		
Final body wt	1.47 [a]	1.41 [b]	1.35 [c]
Length of vertebral column	47.0 [a]	45.7 [a]	44.3 [a]
Adrenals [4]	0.12 [a]	0.13 [a]	0.12 [a]
Heart [4]	6.63 [a]	6.76 [a]	6.46 [a]
Kidneys [4]	6.73 [a]	6.31 [a]	6.49 [a]
Liver [4]	39.1 [a]	35.4 [b]	32.2 [c]
Pancreas [4]	1.62 [a]	1.52 [a]	1.58 [a]
Spleen [4]	2.43 [a]	2.40 [a]	2.27 [a]
Testes [4]	1.31 [a]	1.34 [a]	1.31 [a]

[1] Mean values from 6 pigs. Means with no common superscript letter are significantly different ($P < 0.05$).
[2] Calculated by the least squares method from daily body weights throughout the 14-day experimental period.
[3] Distance between the 6th and 27th vertebrae.
[4] Wet weight of organ, in grams, per kilogram of initial body weight.

The average body weight gain was lowest with the wheat gluten diet and highest with the amino acid diet. These effects did not produce significant differences in final body and organ weights because of wide variations among litters and individuals. Initial body weights varied up to 20% from the litter means. When the data were expressed per unit of initial body weight, the final body and liver weights became significant with lowest values for the wheat gluten group and highest for the amino acid group. The lengths of the vertebral columns, after adjustment for initial body weight, showed a similar trend, but the differences were not significant.

Nitrogen balance data collected during the second week are given in table 4. Since there had been sufficient time for all of the pre-experimental diet to pass out of the digestive tract, live weight gains during this period were probably not due to changes in the amounts of unabsorbed food or water. The larger gains in body weight and weight per gram of nitrogen retained by the amino acid group suggest that more fat was being deposited by this group. A significantly larger energy intake was not observed, probably because of wide individual and daily variations in the amounts of nitrogen-free diet consumed.

Approximately half of the 42.1 g of nitrogen consumed in the 6-day period was retained, but there were highly significant differences ($P < 0.01$) in the retentions by each of the dietary groups (table 4). There were no significant differences in digestibility of the nitrogen (average, 95%). The differences in urinary nitrogen excretion were highly significant for all dietary groups. The higher urinary nitrogen with the wheat gluten diet was accounted for largely by increased urea excretion. The difference between urinary nitrogen excretions in the amino acid and casein-lactalbumin groups was due largely to ammonia. The ratios of total urinary nitrogen and urea nitrogen to total creatinine paralleled the absolute values.

TABLE 4

Nitrogen balance data [1]

	Dietary group		
	Amino acid	Casein-lactalbumin	Wheat gluten
Body wt,[2] kg	9.67 [a]	9.91 [a]	9.41 [a]
Body wt gain,[3] kg	1.67 [a]	1.43 [b]	1.34 [c]
Wt gain/g nitrogen retained	73.7 [a]	58.6 [b]	65.0 [c]
Energy intake, megacalories [4]	14.5 [a]	14.2 [a]	12.8 [a]
Nitrogen retained, g	22.6 [a]	24.2 [b]	20.5 [c]
Fecal nitrogen, g	1.99 [a]	1.74 [a]	2.26 [a]
Urinary nitrogen, g	17.6 [a]	16.2 [b]	19.4 [c]
Urea nitrogen, g	8.2 [a]	8.3 [a]	11.1 [b]
Ammonia nitrogen, g	5.1 [a]	4.1 [b]	4.3 [b]
Urinary creatine, g	0.79 [a]	1.03 [a]	0.71 [a]
Urinary creatinine, g	2.44 [a]	2.56 [a]	2.49 [a]
Creatinine coefficient [5]	55.2 [a]	60.7 [a]	56.0 [a]
Urinary nitrogen:creatinine ratio [6]	5.85 [a]	4.97 [b]	6.72 [c]
Urea nitrogen:creatinine ratio [7]	2.66 [a]	2.56 [a]	3.87 [b]

[1] Six-day collection periods; 6 pigs/group. Means with no common superscript letter are significantly different ($P < 0.05$).
[2] Average of the 7 daily weights for each pig.
[3] Calculated by the method of averages from daily body weights.
[4] Gross energies (kilocalories $\times 10^3$) calculated with the energy factors given in table 2.
[5] Milligrams of total creatinine per day per kilogram body weight (average of 7 daily weights).
[6] Grams of urinary nitrogen per gram of total creatinine (creatine plus creatinine).
[7] Grams of urea nitrogen per gram of total creatinine (creatine plus creatinine).

TABLE 5

Carcass analyses [1]

	Dietary group		
	Amino acid	Casein-lactalbumin	Wheat gluten
Wet wt [2]			
kg	8.40 [a]	8.93 [a]	8.16 [a]
g/g initial body wt	1.16 [a]	1.15 [a]	1.08 [b]
Dry matter			
kg	3.54 [a]	3.46 [a]	3.26 [a]
g/kg initial body wt	488 [a]	448 [b]	434 [b]
% of carcass	41.9 [a]	38.5 [b]	39.7 [b]
g/g carcass nitrogen	20.1 [a]	17.7 [b]	18.1 [b]
Ether extract			
kg	2.10 [a]	1.87 [ab]	1.77 [b]
g/kg initial body wt	286 [a]	239 [b]	233 [b]
% of dry matter	58.4 [a]	53.1 [b]	53.0 [b]
g/g carcass nitrogen	11.88 [a]	9.54 [b]	9.82 [b]
Crude protein [3]			
kg	1.10 [a]	1.21 [b]	1.12 [a]
g/kg initial body wt	152 [a]	158 [a]	150 [a]
% of dry matter	31.7 [a]	36.0 [b]	35.5 [b]
Energy [4]			
megacal [5]	26.0 [a]	24.4 [a]	23.0 [a]
megacal/kg initial body wt	3.55 [a]	3.14 [b]	3.03 [b]
kcal/g dry matter	7.29 [a]	7.02 [b]	6.98 [b]
kcal/g carcass nitrogen	147 [a]	125 [b]	128 [b]

[1] The bled carcasses included head, feet and skin, but no abdominal or thoracic viscera. Six pigs/group. Means with no common superscript letter are significantly different ($P < 0.05$).
[2] After refrigeration for 1–3 days.
[3] N x 6.25.
[4] Calculated on the basis of 9.5 kcal/g of ether extract plus 34.2 kcal/g of body nitrogen (31).
[5] Kilocalories $\times 10^3$.

TABLE 6
Liver analyses [1]

	Dietary group		
	Amino acid	Casein-lactalbumin	Wheat gluten
Dry matter, %	27.2 [a]	26.9 [a]	26.6 [b]
Ether extract			
g	2.90 [a]	3.09 [a]	2.89 [a]
g/kg initial body wt	0.399 [a]	0.404 [a]	0.383 [a]
% of liver wt	1.03 [a]	1.14 [a]	1.21 [a]
g/g liver nitrogen	0.534 [a]	0.537 [a]	0.511 [a]
Crude protein [2]			
g	33.9 [a]	36.1 [a]	34.6 [a]
g/kg initial body wt	4.67 [a]	4.69 [a]	4.61 [a]
% of liver wt	12.1 [a]	13.4 [ab]	14.7 [b]
Non-fat, non-protein dry matter, %	14.1 [a]	12.4 [b]	10.7 [c]
DNA, mg/g liver	0.93 [a]	1.06 [b]	1.06 [b]
Protein, g/mg DNA [3]	32.5 [a]	30.0 [a]	28.5 [a]
RNA:DNA ratio [4]	7.18 [a]	7.44 [a]	8.15 [a]

[1] Six pigs/group. Means with no common superscript letter are significantly different ($P < 0.05$).
[2] N × 6.25.
[3] Protein determined in tissue hydrolysate by the biuret method.
[4] Milligrams ribonucleic acid per milligram deoxyribonucleic acid.

Analyses of the eviscerated carcasses are shown in table 5. Since the wet weights of the eviscerated carcasses follow the trend of the live body weghts, the effects of the amino acid sources on live weight gains were probably not due to differences in fill of the intestinal tract. The chemical analyses of the eviscerated carcasses show that the larger weight gains by the amino acid group were due to fattening. The better growth of the casein-lactalbumin group compared with the wheat gluten group appears to be due primarily to retention of protein and its associated water. These results are consistent with the nitrogen balance data. Since the carcasses in the amino acid group contained more fat, they also had higher energy contents.

Analyses of the livers are shown in table 6. The smaller livers of the wheat gluten group (table 3) contained lower percentages of dry matter. The amounts of ether extract were similar for all groups, whether expressed as total grams, grams per unit of initial body weight, percentage of liver weight, or grams per gram of liver nitrogen. The amounts of crude protein were also similar for all groups when expressed as grams or grams per kilogram of initial body weight but differed when expressed as percentage of liver weight. A trend in the opposite direction occurred in the percentage of dry matter not accounted for by crude protein and ether extract. The amino acid group had a lower concentration of liver DNA. There were no significant differences in the ratios of protein and RNA to DNA.

DISCUSSION

Howe et al. (11) reported equal weight gains by rats pair-fed amino acids or casein supplemented with amino acids to the FAO provisional pattern, but lower gains with cottonseed meal supplemented to the FAO provisional pattern. Studies with young men and women have usually shown higher nitrogen retentions when part of the FAO provisional pattern of amino acids was supplied by eggs, non-fat milk solids, or peanut butter (2, 12). Studies of other amino acid patterns with various species of animals have also usually shown poorer responses with amino acids than with intact proteins. In many of those studies the source of nonessential nitrogen in the amino acid diet differed considerably from that supplied by protein. Also in ad libitum studies the nitrogen intakes may have been affected by energy requirements (13–15),

palatability, or osmotic effects (3). Our finding of less nitrogen retained and more fattening with the amino acid diet than with casein-lactalbumin was made with equal intakes of nitrogen, balanced mixtures of essential and nonessential L-amino acids fed in an agar gel, and energy supplied ad libitum.

A major difference between amino acid and protein diets is the lack of asparagine and glutamine in the former. These amides are absorbed without appreciable hydrolysis and are utilized in the synthesis of protein, transport of ammonia, and other functions. The requirements for these amides can usually be met by synthesis, but if the requirement is increased by a dietary change, 4 to 8 days may be needed by normal rats to produce a compensating increase in the rate of amide synthesis (16, 17). When dietary nitrogen is low this adaptive response may be too slow to meet all requirements. In this case the renal requirement for ammonia to maintain acid-base balance would be expected to take precedence over protein synthesis. Our finding of greater ammonia excretion with the amino acid diet, which contained considerably more amino acid hydrochlorides (table 1), suggests that protein synthesis may have been limited by diversion of glutamine to ammonia production. Renal extraction of blood glutamine occurs in acidotic animals and may be reduced by administration of sodium bicarbonate (18). The beneficial effects of sodium bicarbonate and ammonia precursors on growth of animals fed amino acid diets may be due to sparing actions which make more glutamine and asparagine available for protein synthesis (3, 16, 19, 20).

The extra hydrochlorides in our amino acid diet may also have stimulated fattening. Lotspeich (17) reported that acidosis induced by feeding ammonium chloride or lysine hydrochloride increased the activity of hexose monophosphate shunt enzymes in the kidney. The shunt system produces NADPH, which stimulates synthesis of fatty acids and glycogen (via the dicarboxylic acid shuttle). Isotopic tracer experiments showed that the gluconeogenesis utilized the carbon skeleton of glutamine. Lotspeich suggested that this function increased the renal extraction of glutamine beyond the requirements for increased excretion of ammonia.

Radiocarbon tracer studies by Ahrens et al. (13, 14) also showed a shift from Embden-Meyerhof glycolysis to alternate pathways when amino acids were substituted for casein. They stated (13), that "When the nitrogen intake was low and the calories were high, there was a trend toward higher storage of non-protein calories (fat) when the source of nitrogen was amino acids rather than casein." That trend was not statistically significant, but effects of nitrogen sources on fattening may be apparent only in animals that are rapidly producing adipose tissue. Our finding, that replacing protein with amino acids increased fattening in pigs supplied energy ad libitum, may be more nearly applicable to human situations than results obtained with rats fed diets that have fixed calorie-to-nitrogen ratios.

In summary, our data for the amino acid group are consistent with the hypothesis that there was insufficient glutamine and asparagine from exogenous and endogenous sources to meet the requirements for protein synthesis, ammonia excretion and, perhaps, glycogen synthesis. When the pigs were fed the amino acid diet, they were deprived of their exogenous source of glutamine. Since this diet contained more amino acid hydrochlorides, the kidneys extracted more glutamine from the blood to produce ammonia. With only a low level of nitrogen in the diet, the adaptive response to increase glutamine synthesis was too slow. Some acidosis developed, which stimulated synthesis of fatty acids and glycogen. The gluconeogenesis may have further depleted the supply of glutamine. The lack of glutamine retarded protein synthesis (nitrogen retention) and thus made more nitrogen available for ammonia excretion. A similar situation was not produced by the casein-lactalbumin diet because it continued to supply some glutamine and asparagine and it also contained less amino acid hydrochlorides.

Our observation of greater weight gains per gram of nitrogen retained by the wheat gluten group than by the casein-lactalbumin group is similar to the findings of Allison with dogs (21) and Hegsted and Chang with rats (22). However, analyses

of the eviscerated carcasses of our pigs did not show significantly larger amounts of dry matter or fat per gram of nitrogen.

Impaired utilization of wheat gluten nitrogen by our pigs was shown by nitrogen balance and weight gains. This conclusion was further supported by the ratios of urinary nitrogen and urea to creatinine, and by the final weights (adjusted for differences in initial body weight) of the live animals, eviscerated carcasses, and livers. Several other measurements also suggested impaired utilization, but with less than half of the dietary nitrogen supplied by the test proteins, only 6 animals per test group, and wide individual variation, they were not statistically significant. The nitrogen-balance data show that digestion and absorption of total nitrogen in wheat gluten was not impaired, but these data do not reveal effects on individual amino acids. The larger amounts of urea nitrogen produced from the wheat gluten diet could be due to faulty digestion, absorption, or intermediary metabolism of a specific amino acid, with increased catabolism of the residual unbalanced pattern of amino acids. Degradation of poorly utilized proteins by bacteria in the small intestine of young, protein-depleted pigs has been postulated by Barnes et al. (23).

Calhoun et al. (24) reported that supplementation of a lysine-deficient diet with wheat gluten produced lower nitrogen gains in weanling rats than did supplementation with pure lysine equal to the lysine content of the wheat gluten. While incomplete availability of the lysine in wheat gluten was suggested, the imbalance of amino acids in this protein may have been a contributing factor (25). Similar studies suggested incomplete availability of valine and isoleucine (26). Since the process by which their wheat gluten had been prepared was not given, the extent of heat injury (Maillard reaction) during drying cannot be evaluated.

The vacuum-dried wheat gluten used in our study showed a high level of total amylase activity when assayed by a modification of the ICC method (27). The presence of active enzyme suggests that our protein had not been greatly altered by processing. The finding that this highly digestible wheat gluten protein was incompletely utilized by weanling pigs consuming a balanced pattern of amino acids suggests that, in designing cereal diets for the prevention of kwashiorkor, the availability of amino acids should be considered.

The major difference observed in the livers was in the non-fat, non-protein fraction of dry matter. This fraction included carbohydrate, ash, some phospholipids, and probably some lipids oxidized during the drying (101° air oven for 16 to 18 hours) before ether extraction. High glycogen values in the livers of protein-deficient pigs were reported by Platt et al. (28) and Schneider and Sarett (29). The data given by both groups of workers show higher glycogen levels in pigs that were depositing more fat. The data of Filer et al. (30) also show that a low protein, fattening diet produced livers that contained more carbohydrate (calculated by difference). Glycogen determinations were not made in our experiment because the livers remained in the carcasses for variable lengths of time before freezing. Whether hydrolyzed or not, glycogen would appear in the non-fat, non-protein fraction. The higher percentage of non-fat, non-protein dry matter in the livers of our amino acid groups may have been caused by an increase in glycogen or unextracted lipids associated with the fattening induced by the hydrochlorides in this diet. This increase in dry matter accounts for the lower percentages of protein and DNA in the livers of these animals. The differences in non-fat, non-protein dry matter, plus water associated with it, probably also explains the differences in relative liver weights (table 3).

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Time, Stress, Quality, and Quantity as Factors in the Nonspecific Nitrogen Supplementation of Corn Protein for Adult Men [1]

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ABSTRACT Quantity, quality, stress imposition, and prolonged feeding time were investigated as possible influencing factors in the "nonspecific" nitrogen supplementation of corn for human consumption. In study 1, mean nitrogen balances of 8 men fed 6.0 g nitrogen from white, degerminated corn meal plus supplements of 2.0 or 6.0 g nitrogen/day from milk, gelatin, soybean meal, zein, or a glycine-diamonium citrate mixture were: +1.11 and +3.22 g nitrogen (milk), +0.63 and +2.14 (gelatin), +0.83 and +3.21 (soybean meal), +0.54 and +2.00 (zein), and +0.25 and +1.15 (glycine-diammonium citrate), respectively. In study 2, nitrogen balances of 10 adult men showed no consistent changes over a 57-day period when a constant diet providing 6.0 g nitrogen from corn meal and 6.0 g nitrogen from a glycine-diammonium citrate mixture was fed. Following application of a physical stress (loss of approx. 473 ml of blood), 5 subjects continuing to receive the "nonspecific" nitrogen-supplemented corn diet showed a sharp increase in nitrogen retention which tended to return to former levels. In contrast, 5 subjects who were changed from the "nonspecific" nitrogen-supplemented corn diet to a similarly supplemented milk diet, following stress application, showed a sharper increase in nitrogen retention and maintained a higher degree of nitrogen retention until termination of the study.

Information from this and other laboratories indicates that level of dietary "nonspecific" nitrogen, that is, nitrogen from any metabolically usable, nontoxic source, may be an important factor in determining minimal protein need for humans (1–7).

In the current project, 4 aspects of "nonspecific" nitrogen supplementation of corn, a comparatively poor quantity-quality source of protein, were studied. These included determination of the effect on nitrogen retention of adult men of 1) variation in source of "nonspecific" nitrogen supplementation of corn; 2) variation in level of "nonspecific" nitrogen supplementation of corn; 3) feeding a constant, "nonspecific" nitrogen-supplemented corn diet over a relatively long period of time; and 4) imposition of a physical stress when "nonspecific" nitrogen-supplemented corn diets or "nonspecific" nitrogen-supplemented milk diets were fed.

PROCEDURE

Study 1. The first study (of the 2 studies in the project) consisted of 2 parts, designated as part A and part B. Each 33-day part included an introductory 3-day nitrogen depletion period, a 5-day nitrogen adjustment period, and 5 experimental periods of 5 days each arranged at random for each subject. The 2 parts followed one another directly with no lapse in time and used the same subjects. Subjects 81, 83, 85, 87, and 89 first followed the regimen imposed by part A followed by that imposed by part B, while subjects 84, 86, and 90 first followed the regimen of part B followed by that of part A.

During the preliminary nitrogen depletion period of both parts A and B, nitrogen intake per subject per day totaled 2.5 g; that is, 2.00 g nitrogen from white, degerminated corn meal [3] and 0.50 g from the basal diet as described in table 1. Purposes of this period included the utilization of a low nitrogen diet to speed the adjust-

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[3] The corn meal used in these studies was kindly supplied by the Gooch Milling and Elevator Company, Lincoln, Nebraska.

TABLE 1

Diet plan

Period	No. days	Principal dietary protein		Nitrogen supplement		Total nitrogen intake [2]
		Corn [1]	Milk	Source	Amount	
		g N/day			*g N/day*	*g N/day*
Study 1						
Part A [3]						
Depletion	3	2.0	—	none	—	2.5
Adjustment	5	6.0	—	varied [4]	2.0	8.5
Exp. 1	5	6.0	—	milk [5]	2.0	8.5
Exp. 2	5	6.0	—	gelatin [6]	2.0	8.5
Exp. 3	5	6.0	—	soybean meal [7]	2.0	8.5
Exp. 4	5	6.0	—	zein	2.0	8.5
Exp. 5	5	6.0	—	DAC + glycine [8]	2.0	8.5
Part B [3]						
Depletion	3	2.0	—	none	—	2.5
Adjustment	5	6.0	—	varied [4]	6.0	12.5
Exp. 6	5	6.0	—	milk [5]	6.0	12.5
Exp. 7	5	6.0	—	gelatin [6]	6.0	12.5
Exp. 8	5	6.0	—	soybean meal [7]	6.0	12.5
Exp. 9	5	6.0	—	zein	6.0	12.5
Exp. 10	5	6.0	—	DAC + glycine [8]	6.0	12.5
Study 2						
Depletion	3	2.0	—	none	—	2.5
Adjustment	7	6.0	—	DAC + glycine [8]	6.0	12.5
Exp. 1	10	6.0	—	DAC + glycine	6.0	12.5
Exp. 2	10	6.0	—	DAC + glycine	6.0	12.5
Exp. 3	10	6.0	—	DAC + glycine	6.0	12.5
Exp. 4	10	6.0	—	DAC + glycine	6.0	12.5
Exp. 5	10	6.0	—	DAC + glycine	6.0	12.5
Stress						
Exp. 6	5	6.0	or 6.0 [9]	DAC + glycine	6.0	12.5
Exp. 7	5	6.0	or 6.0	DAC + glycine	6.0	12.5
Exp. 8	5	6.0	or 6.0	DAC + glycine	6.0	12.5
Exp. 9	5	6.0	or 6.0	DAC + glycine	6.0	12.5

[1] White, degerminated corn meal having a nitrogen content of 0.01316 g N/g meal (study 1) or 0.01157 g N/g meal (study 2) was used.

[2] Total nitrogen intake figures include the 0.50 g N/day provided by the basal diet. The basal diet consisted of 100 g applesauce, 3.5 g dry boullion, 50 g cabbage, 100 g peaches (alternated daily with 100 g apricots in study 1), 100 g pears (alternated daily with 100 g pineapple in study 1), 90 g jelly, and 10 g dry instant decaffeinated coffee. A vitamin supplement provided 5000 IU of vitamin A (synthetic), 500 IU vitamin D (900 IU vitamin D in study 2); and (in milligrams) thiamine, 2; riboflavin, 2.5; ascorbic acid, 50; pyridoxine, 1; niacin, 20; Ca pantothenate, 1; and vitamin B_{12}, 1 μg. Mineral supplements provided the following per subject per day: (in grams) Ca, 1.00; P, 1.00; Mg, 0.199; Fe, 0.015; Cu, 0.002; K, 0.00005; I, 0.00015; Mn, 0.002; and Zn, 0.0009. NaCl was allowed ad libitum.

[3] Subjects 81, 83, 85, 87 and 89 on part A first, then part B. Subjects 84, 86, 90 on part B first, then part A. Order of experimental periods 1 through 5 and 6 through 10 randomized for each subject.

[4] Sources of nitrogen supplement during adjustment periods were varied among the subjects so as to be the same as that which each received during the first experimental period of part A and Part B.

[5] Dry, skim milk powder having a nitrogen content of 0.0542 g N/g powder was used.

[6] Dry, gelatin powder having a nitrogen content of 0.1572 g N/g powder was used.

[7] Low fat, soybean flour having a nitrogen content of 0.07843 g N/g flour was used.

[8] An isonitrogenous mixture of glycine and diammonium citrate was used.

[9] After stress, subjects 101–105 continued to receive corn as principal dietary protein while the chief source of dietary protein for subjects 106–110 was changed to dry, skim milk powder.

ment of subjects to later experimental diets (introduction of subjects to their duties and responsibilities) and, together with the following adjustment period, determination of individual caloric requirements for weight maintenance.

During the adjustment period and experimental periods of both parts A and B, nitrogen intake from white degerminated corn meal was maintained constant at 6.0 g per subject per day. Milk, gelatin, soybean flour, zein or a mixture of glycine

and diammonium citrate provided either 2.0 g nitrogen per day during these periods in part A or 6.0 g nitrogen per subject per day during the adjustment and experimental periods in part B. These supplementary sources of nitrogen were selected as representative of both good and poor quality protein types. Thus, with the inclusion of 0.50 g nitrogen provided by the basal diet, total nitrogen intake during the adjustment and experimental periods was maintained at 8.50 g per day in part A and 12.50 per day during part B. The adjustment period was included in both parts to allow time for subjects to reach a stabilized nitrogen output on the particular nitrogen intake level involved before imposing the experimental variables. Diets used during both parts A and B of study 1 are described in table 1.

Study 2. The second study of the project was also composed of 2 parts, part A which was 60 days long and part B, 20 days long. The same subjects were used in both, and part B followed part A directly with no time lapse.

Part A was composed of a 3-day nitrogen depletion period, a 7-day nitrogen adjustment period and 5 experimental periods of 10 days each. During the 3-day nitrogen depletion period, total nitrogen intake was maintained at 1.50 g per subject per day, 1.0 g nitrogen provided by white, degerminated corn meal and 0.50 g nitrogen from the basal diet (table 1). Objectives of this period were as described under study 1. To determine the effects of prolonged feeding of a "nonspecific" nitrogen-supplemented corn diet to adult humans, a constant diet was fed throughout the adjustment and experimental periods of part A which provided a total of 12.50 g nitrogen per day, 6.0 g nitrogen from white degerminated corn meal, 6.0 g nitrogen from an isonitrogenous mixture of glycine and diammonium citrate, and 0.50 g nitrogen from the basal diet (table 1).

On the morning of day 61 of study 2 (the first day of part B), a physical stress in the form of the loss of one pint of blood (approx. 473 ml as a donation to the Red Cross) was imposed on the subjects. Subject 102 did not receive this stress. Half of the subjects (101, 102, 103, 104, 105) continued to receive the glycine-diammonium citrate-supplemented diet described in part A for the remaining 20 days of the study, while the other half (subjects 106, 107, 108, 109, 110) consumed a constant diet which also provided 12.5 g nitrogen per subject per day but furnished by 6.0 g nitrogen from skim dry milk powder, 6.0 g nitrogen from glycine and diammonium citrate and 0.50 g nitrogen from the basal diet. The 20 days of part B were divided into 4 periods of 5 days each.

Subjects. Details regarding the men, inmates of the Nebraska Penal and Correctional Complex, who were subjects for studies 1 (8 men) and 2 (10 men) are listed in table 2. Institutional health records, as well as physical examinations conducted at the beginning and end of the project and observations made by the institution's physician indicated that all were in good health. Subjects were housed and ate meals together in a separate part of the institution; however, usual work assignments were maintained.

Diets. In study 1, the daily allotment for each subject of corn meal was combined with various levels of cornstarch [4] and with dry skim milk solids, powdered gelatin, enriched white wheat flour, or soybean meal as demanded by the experimental design (table 1) so that the total from all of these sources equaled 600 g per subject per day. These allotments were mixed with a calcium phosphate baking powder, fat, mineral supplement, cellulose flakes, sodium chloride, and water using a basic muffin method. The dough was divided into thirds by weight for consumption at each of the daily meals, shaped into drop biscuits on aluminum foil and baked. Extra-calorie cornstarch-fat wafers were prepared basically by the same method.

In study 2, the daily allotment of corn meal or of dry skim milk powder for each subject was combined with wheat starch [5] in amounts so as to total 600 g from all sources. To these were added fat, sucrose, sodium chloride, glycerol monostearate, pure glycerol, dry yeast, mineral mix, cellulose flakes and water and prepared basically according to the method presented by

[4] The corn oil and cornstarch used in these studies was kindly supplied by Corn Products Company, Argo, Illinois.

[5] The wheat starch used in these studies was kindly supplied by the Hercules Powder Company, Inc., Harbor Beach, Michigan.

TABLE 2

Age, height, weight, and caloric intakes of the subjects

Subject no.	Age	Height	Weight		Calories
			Beginning	Final	
	years	*cm*	*kg*	*kg*	*kcal/kg/body wt/day*
		Study 1			
81	27	174	82	80	44
83	22	183	75	74	53
84	26	180	71	71	55
85	21	175	85	84	38
86	27	173	84	84	50
87	22	183	72	72	47
89	25	175	78	78	50
90	26	175	89	89	40
		Study 2			
101	22	180	73	68	55
102	21	173	71	68	51
103	22	178	79	78	46
104	39	179	81	81	39
105	25	165	82	82	44
106	21	183	74	75	49
107	28	187	90	90	40
108	23	180	75	75	36
109	33	175	78	78	35
110	26	173	68	68	53

Steele et al. (8). These yeast-risen biscuits were fed in equal amounts by weight at each of the three daily meals. Extra-calorie wheat starch-fat muffins were prepared basically by the same method (8).

In both studies, caloric intake for each individual was maintained constant at the level required for approximate weight maintenance (table 2) by varying the intake of starch and fat among the subjects; however, in all cases, fat provided 20% of the calories. Fat sources used in the 2 studies were butter fat and corn oil.[6]

In both studies, glycine-diammonium citrate mixtures when used were presented to subjects in water solutions at each of the 3 meals in equal amounts. A nonprotein, low calorie carbonated beverage [7] was allowed ad libitum. Other diet items, including vitamin and mineral supplements, were as described in table 1.

Analyses. The nitrogen balance technique was used as the principal method of evaluation. Urine, feces, and food were collected and prepared for analysis as described previously and were analyzed for nitrogen content by the boric acid modification of the Kjeldahl method (9). Urine samples were preserved under toluene and were analyzed daily for 24-hour excretion of nitrogen and creatinine (10). Daily fecal nitrogen values were based on 5- or 10-day fecal composites as determined by the length of the experimental periods involved.

Fasting venous blood samples were collected from each subject on days zero (previous to start of study), 21, 41, 62 (day following loss of one pint of blood), 65, 71, and 81 of study 2 which were analyzed by a hospital laboratory [8] via routine clinical procedures (table 3) as a screening device for possible gross abnormalities.

RESULTS

Mean nitrogen balances of subjects receiving corn diets supplemented at 2 levels with various sources of nitrogen are shown

[6] Subjects 81, 83, 84, 85 in all periods of study 1 and subjects 102, 104, 106, 108, 110 during the adjustment period through period 2, and subjects 101, 103, 105, 107, 109 during periods 3–9 of study 2 received butter oil, while subjects 86, 87, 89, 90 of study 1 during all periods and subjects 101, 103, 105, 107, 109 during adjustment period through period 2, and subjects 102, 104, 106, 108, 110 during periods 3–9 of study 2 received corn oil.

[7] The nonprotein, low calorie carbonated beverages used in these studies were kindly supplied by the Pepsi Cola Company, New York, and the Pepsi Cola Company, Lincoln, Nebraska.

[8] These analyses were made by St. Elizabeth Hospital Laboratory, Lincoln 2, Nebraska; E. D. Zeman, M.D., and J. F. Porterfield, M.D., pathologists.

TABLE 3

Effect of constant level and "nonspecific" nitrogen supplementation of diets as influenced by length of time and stress

Determination [1]	Group [2]	Mean fasting blood value measured on day [3]						
		0	21	41	(61) (Blood loss stress)	62	71	81
Hematocrit, ml/100 ml	1	47	47	47		42	41	44
	2	50	49	50		47	49	49
Hemoglobin, g/100 ml	1	15.6	15.3	15.6		14.6	14.9	15.3
	2	16.6	16.0	16.4		16.0	16.5	16.4
Sedimentation rate	1	8	5	7		10	9	11
	2	4	4	4		9	6	7
White blood count, ($mm^3 \times 10^3$)	1	8.1	8.5	5.9		6.3	6.5	6.7
	2	7.9	9.8	9.0		6.0	6.6	6.5
Segmented polymorphonuclear leukocytes	1	46	50	54		47	45	54
	2	51	51	53		57	55	51
Lymphocytes, %	1	43	43	40		44	46	36
	2	43	42	41		38	36	39
Monocytes, %	1	4	3	4		6	2	4
	2	3	5	4		3	4	5
Glucose, mg/100 ml	1	97	99	95		92	89	97
	2	98	94	97		99	94	99
Blood urea N, mg/100 ml	1	11	11	13		12	11	11
	2	10	12	13		15	13	11
Serum glutamic pyruvate transaminase, Babson units	1	25	34	31		27	10	22
	2	22	28	34		19	3	16
Total protein, g/100 ml	1	7.0	7.3	6.8		6.4	6.6	6.8
	2	6.7	7.4	6.5		6.4	6.8	6.7
Albumin, g/100 ml	1	4.5	4.4	4.5		4.4	4.2	4.3
	2	4.5	4.5	4.4		4.5	4.4	4.4
Globulin, g/100 ml	1	2.5	2.9	2.9		2.0	2.4	2.5
	2	2.2	3.1	2.1		1.9	2.3	2.3
Albumin/globulin ratio	1	2.0	1.6	2.0		2.2	1.8	1.8
	2	2.1	1.6	2.1		2.5	1.9	1.9

[1] Determinations were by standard laboratory procedures of the St. Elizabeth Hospital Laboratory, Lincoln, Nebraska. Additional information regarding methodology and quality control (within 1% for method used) is available from this laboratory.

Hematology methods: a) erythrocyte sedimentation rate and hematocrit by the Wintrobe Hematocrit tube method under standard conditions as described in Wintrobe Clinical Hematology, ed. 4. Lea and Febiger, Philadelphia; b) leukocyte and erythrocyte counts by Coulter Counter (Model F); c) hemoglobin determination using cyanmethemoglobin stable reagent (NIH and Sunderman Standards) read in Coleman Junior Spectrophotometer at 550 mμ setting; and d) differential counts by examination of 100 cells in representative fields stained by the Wright stain procedure.

Autoanalyzer methods: a) glucose by a modification of the W. S. Hoffman method (J. Biol. Chem., *120:* 51, 1937); b) urea nitrogen by a modification of the L. T. Skeggs method (Amer. J. Clin. Pathol., *28:* 311, 1957); c) total protein by the T. E. Weichselbaum modification of the D. L. Stevens method (Amer. J. Clin. Pathol., *7:* 40, 1946); and d) albumin by the method of D. D. Rutstein, E. T. Ingenito and W. E. Reynolds (J. Clin. Invest., *33:* 211, 1954).

[2] Subjects in group 1 (101–105) and group 2 (106–110) received identical diets for the first 60 days of the study which provided 6.0 g N from corn plus 6.0 g N from glycine and diammonium citrate. From day 60 through day 80, group 1 subjects continued to receive this diet while group 2 subjects received a similar diet in which corn protein was isonitrogenously replaced with milk. Data for subject 102 who was not subjected to blood loss stress is not included.

[3] Venous blood samples were drawn in the morning before consumption of the first meal of the day. Day zero fasting blood samples were drawn 3 days before the start of the study.

in figure 1. Results indicated an increase in nitrogen retention resulting from increasing "nonspecific" nitrogen supplementation from 2.0 to 6.0 g per day regardless of source. Mean differences in nitrogen balances between these 2 levels of supplementation were 2.11 g nitrogen per day with milk, 1.51 g nitrogen per day with gelatin, 2.38 g nitrogen per day with soybean meal, 1.46 g nitrogen per day with zein, and 0.90 g nitrogen per day with the glycine-diammonium citrate mixture. These differences were statistically significant at the 0.05% level. However, nitrogen retention varied among the supplements used at both levels of total nitrogen intake. At the 8.5-g total nitrogen intake level (6.0 g nitrogen from corn plus 2.0 g nitrogen from supplement), the milk addition resulted in mean nitrogen balance of +1.11 g nitrogen per day which was statistically higher than the mean nitrogen balances of +0.63, +0.83, +0.54, +0.25 achieved when gelatin, soybean flour, zein, and the glycine-diammonium citrate mixture were added, respectively. At the 12.5-g total nitrogen intake level (6.0 g nitrogen from corn plus 6.0 g nitrogen from supplement), the milk supplement and the soybean supplement resulted in mean nitrogen balances of +3.22 and +3.21 g nitrogen per day, respectively, which were statistically higher than the mean nitrogen balances of +2.00 and +2.14 g nitrogen per day achieved with the zein and gelatin, respectively; these in turn were statistically higher than the mean nitrogen balance of +1.15 g nitro-

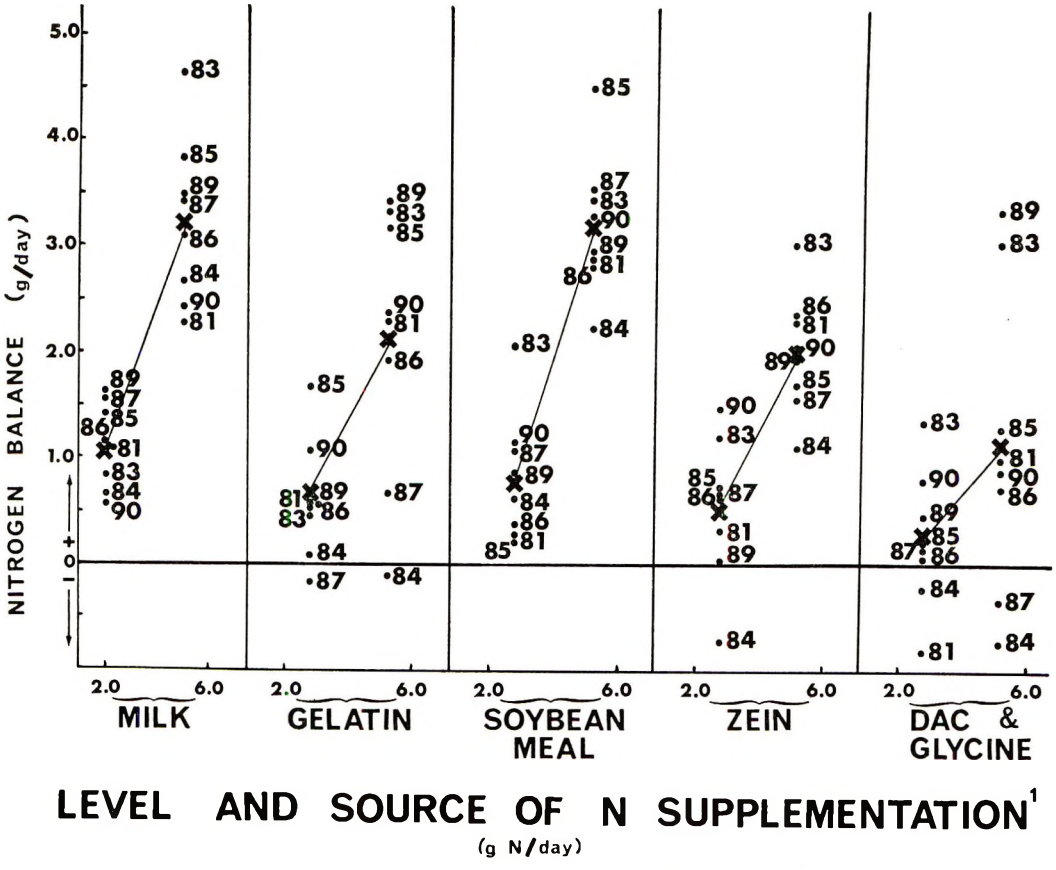

Fig. 1 Nitrogen balances of adult men fed a standardized corn diet (6 g N/day) supplemented at 2 levels with various sources of additional nitrogen. Legend: × = mean balances for all subjects; ● = individual subject.

gen per day obtained with glycine-diammonium citrate supplementation.

Mean nitrogen balances and blood analysis data of subjects fed 6.0 g nitrogen from corn plus 6.0 g nitrogen from a mixture of glycine and diammonium citrate for 57 days are shown in figure 2 and table 3. Following initial nitrogen depletion and adjustment periods, no correlations between any of these biochemical measurements and length of time that the experimental diet was fed were found.

Data obtained from the same subjects following the administration of a physical stress are shown in figure 2 and table 3. Those subjects who continued to receive the corn plus glycine-diammonium citrate diet showed a mean daily increase of 0.96 g nitrogen in the 5 days immediately following stress application. However, in the next three 5-day periods, nitrogen retention slowly decreased. During the final period, an average of only 0.42 g more nitrogen was stored per day than during the pre-stress periods.

However, those subjects who were changed to a glycine-diammonium citrate-supplemented milk diet showed a mean daily increase in nitrogen balance of 1.50 g in the 5 days immediately following stress application. As in the case of the subjects receiving the corn-supplemented diets, nitrogen retention slowly decreased in the following 15 days. But, in the final period,

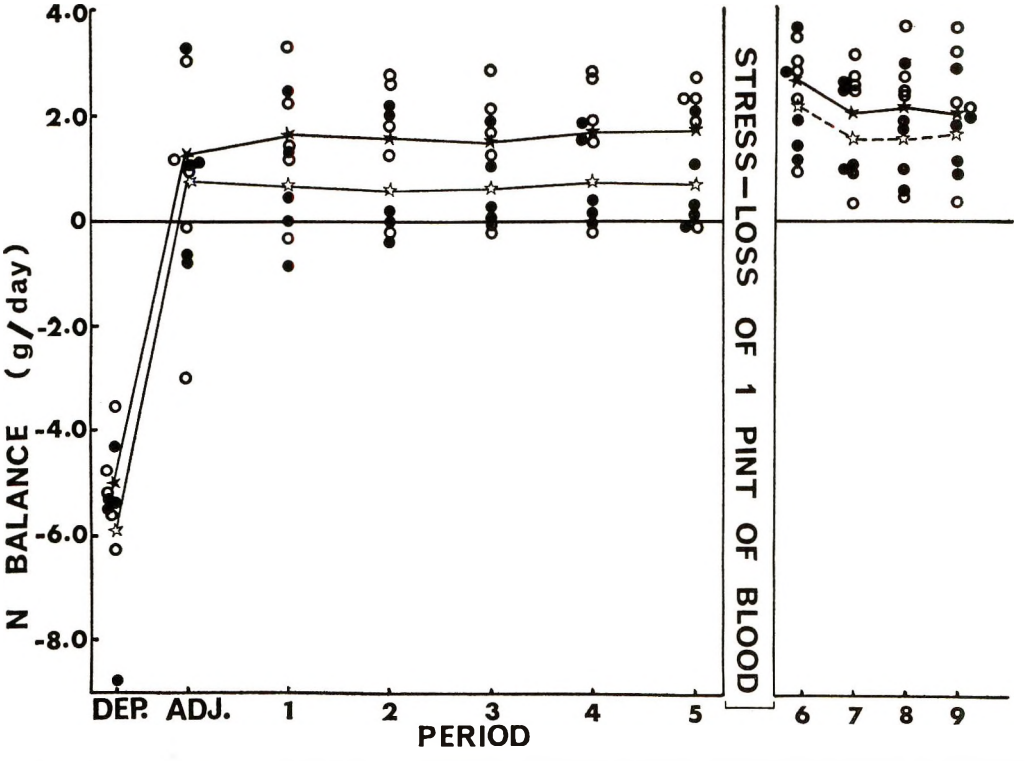

Fig. 2 Nitrogen balances of adult men fed nonspecific nitrogen-supplemented corn diets; influence of prolonged feeding, stress, change to milk diet. Subjects in the 2 groups received identical corn-nonspecific nitrogen-supplemented diets during the depletion period (2.5 g N/day for 3 days), the adjustment period (7 days), the experimental periods 1–5 (12.5 g N/day during 10-day periods); however, during experimental periods 6–9 (5-day periods), group 1 subjects continued to receive 6.0 g N from corn plus 6.0 g N from diammonium citrate and glycine while group 2 subjects were changed to a diet providing 6.0 g N from milk plus 6.0 g N from glycine-diamonium citrate. On day 61 of the study, subjects (except 102) each lost one pint (approx. 473 ml) of blood (physical stress agent). Data for subject 102 not included in mean values for periods 6–9. Legend: ★ = mean and ● = individual nitrogen balances of group 1; ✪ = mean and ○ = individual nitrogen balances of subjects of group 2.

the subjects receiving the supplemented milk diet were still retaining an average of 1.07 g more nitrogen per day than during the pre-stress period when the corn supplemented diet was given.

DISCUSSION

Glycine-diammonium citrate supplementation of suboptimal corn diets has been shown in this and earlier studies (4–6) to be effective in the improvement of nitrogen retention by adult men. Similar results have been observed with the glycine-diammonium citrate supplementation of suboptimal rice diets in adults (7) and with the urea or glycine supplementation of milk diets for human infants (1).

True, long-term, laboratory-controlled nutrition studies in relation to total lifespan of human subjects are impossible. Thus, the permanence of the observed effect on human subjects fed any experimental diet is difficult to ascertain. In the current project, however, the failure of occurrence of any consistent change in apparent nitrogen retention (or in other measured parameters) of adult men over a 57-day period during which a constant glycine-diammonium citrate-supplemented corn diet was fed appears to indicate a relative stability. Conversely, explanations for the apparent sparing action of "nonspecific" nitrogen intake on essential amino acids based on temporary mechanisms thus must be eliminated. The apparent storage of nitrogen denoted by consistently positive nitrogen balances is difficult to explain. This occurrence is frequently noted in laboratory nitrogen balance studies and may be related in part to the fact that all nitrogen losses are not measured in the standard procedure. While this explanation appears to be inadequate, the need for establishment of individual baselines for nitrogen equilibrium rather than the simple, arbitrary use of negative or positive balance seems indicated until a more adequate solution is found.

Another difficulty in application of conclusions based on the results of controlled feeding trials with humans to field situations is that it is impractical to attempt to duplicate all possible variables of physiology and environment of ordinary life to each experimental diet in the controlled laboratory setting. In the laboratory situation, these variables are eliminated or controlled to the greatest possible extent. In the present project, even the mild physical stress of loss of one pint of blood produced a pronounced increase in nitrogen retention. In other studies, physiological-psychological factors such as loss of sleep, night-day reversal in the sleep pattern, or mental stress of examinations have been shown to affect nitrogen balances of human adults (11). While it is impossible to separate all possible physiological-psychological stress factors involved in the loss of one pint of blood, the results of the current project indicate that the specific glycine-diammonium citrate-supplemented corn diet permits an increase in nitrogen retention in adult men as a result of stress or for protein repletion following blood loss, or both.

In this laboratory, "nonspecific" nitrogen supplementation of corn diets has been accomplished using a mixture of glycine-diammonium citrate as the source of supplemental nitrogen (4–6). A "nonspecific" nitrogen addition such as this allows for increase in total nitrogen intake without influencing the essential amino acid proportionality pattern or increasing the intake of other nutrients which may be present in suboptimal amounts in the basal diet. By addition of both "good" and "poor" quality proteins at 2 levels as sources of "nonspecific" nitrogen supplements to a corn diet, the essential amino acid proportionality pattern as well as total nitrogen intake were affected. Precise comparison of glycine-diammonium citrate supplements with the intact protein supplements are difficult since several studies indicate that ingestion of purified amino acid sources of nitrogen results in lower nitrogen retention than when similar intact proteins are fed (12, 13).[9] In addition, the forementioned variations in intake of other nutrients resulting when natural foods are used as supplement sources may also influence results. However, even when the essential amino acid proportionality patterns were possibly adversely affected as with the zein or gelatin additions,

[9] Kies, C. Studies in urinary nitrogen excretion. II. Comparison of the utilization of intact egg protein and pure amino acids. M.S. Thesis, University of Wisconsin, 1960.

the observed increase in nitrogen retention was even greater than that achieved by glycine-diammonium citrate at the highest level tested. The greater degree of nitrogen retention achieved with soybean and milk additives is indicative that the maximum in apparent nitrogen retention was probably not obtained with the "lower quality" additives. The reasons for, or even the desirability of, high levels of apparent nitrogen retention in human adults have not been defined. Thus, it still remains a question whether the similar levels of nitrogen retention observed on feeding suboptimal corn diets supplemented at low levels with milk or at high levels with glycine-diammonium citrate really represent similar states of protein nutriture.

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Effect of Protein Source and Feeding Method on Zinc Absorption by Calves [1]

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ABSTRACT The effects of isolated soybean protein on ^{65}Zn absorption in calves when the protein was delivered to the rumen by mixing in the grain ration or to the abomasum by feeding in milk through nipple pails were investigated. Four 92-kg calves fed alfalfa hay and a concentrate mixture containing cottonseed meal were given 40 μCi ^{65}Zn daily in milk through nipple pails for 14 days. During the last 6 days, net ^{65}Zn absorption for 2 calves receiving 100 g isolated soybean protein (ISP) daily in milk averaged 26% compared with 40% for 2 controls. In a second experiment, an additional 4 calves weighing 93 to 140 kg were paired according to weight and dosed daily with 40 μCi ^{65}Zn in gelatin capsules. They were fed a ration containing 54% corn meal, 39.5% beet pulp, 5% molasses, and 0.5% each of salt, $CaHPO_4$, and $CaCO_3$. During the last 5 days of two 7-day periods of a reversal trial, each calf received 100 g daily of ISP or casein mixed in the feed. Net ^{65}Zn absorptions averaged 28 and 30%, respectively, when ISP and casein were fed. However, when protein sources were fed in milk through nipple pails, net ^{65}Zn absorptions averaged 19 and 34%. Thus, when soy protein was delivered to the abomasum, zinc availability to calves was reduced as has been observed previously in monogastric animals. The effect of soy protein was not evident anterior to the cecum and was not explained by differences in gastrointestinal distributions of ^{65}Zn or binding of ^{65}Zn to undigested residues in the tract.

Zinc deficiency, which can occur under field conditions in a number of monogastric species, including man (1), has been experimentally induced in ruminant animals by feeding semipurified diets very low in the element (2–4). With the exception of conditions in cattle responding to zinc supplementation in British Guiana (5) and in Finland (6), problems with cattle under practical conditions have not been reported. Apparently, much of the problem with swine and poultry stems from low zinc availability in diets containing plant protein sources (7, 8). O'Dell et al. (9) have postulated that a 3-way interaction among calcium, zinc, and phytate forms a complex less available than is formed by any two of the ions alone. Hartmans (10), after an extensive literature review, concluded that such an interaction is unlikely in ruminants. Sesame meal, which has produced severe zinc deficiency in chicks (11), had no observable adverse effects when fed to calves (12). Phytin phosphorus is essentially as available to ruminants as that supplied by monocalcium phosphate (13). Hydrolysis of phytin in the rumen (14) would most likely destroy its zinc binding capacity. However, if the rumen were by-passed and feed delivered to the abomasum, ruminants might behave like monogastric animals with respect to zinc absorption. This can be accomplished in calves by feeding warm milk through a nipple pail (15). Thus, if soybean protein were included in calf milk replacers,[3] availability of dietary zinc could assume practical importance for nursing calves.

EXPERIMENTAL PROCEDURE

Eight calves were used in 2 experiments to study the effects of isolated soybean protein on apparent zinc absorption (table 1). All animals were confined in metabolism stalls for radioisotope administration and total fecal collection. In the first experiment, four 3-month-old calves averaging 92 kg were fed alfalfa hay and a concentrate mixture twice daily at 8 AM and

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[2] Operated by the Tennessee Agricultural Experiment Station for the U.S. Atomic Energy Commission under Contract no. AT-40-1-GEN-242.

[3] Schmutz, W. G., W. W. Cravens, W. L. Soldner and D. L. Hughes 1967 Evaluation of a soybean protein concentrate in calf milk replacers. J. Dairy Sci., *50:* 993 (abstract).

TABLE 1
Age, body weight, and treatment of calves

Calf number	1	2	3	4
Age, months	4.0	3.0	3.5	3.3
Body wt, kg	86	95	89	98
Day of dosing	Supplemental protein source			
	^{65}Zn given in milk (exp. 1)			
1–6	none	none	none	none
7–14	soybean	soybean	none	none

Calf number	5	6	7	8
Age, months	7.3	6.5	5.7	4.8
Body wt, kg	115	110	109	93
Day of dosing	Supplemental protein source			
	^{65}Zn given by capsule (exp. 2)			
1–4	none	none	none	none
5–11	casein in conc	soybean in conc	soybean in conc	casein in conc
12–18	soybean in conc	casein in conc	casein in conc	soybean in conc
19	none	none	none	none
20–27	casein in milk	soybean in milk	soybean in milk	casein in milk
28–37	soybean in milk	casein in milk	casein in milk	soybean in milk

TABLE 2
Apparent ^{65}Zn absorption as determined by total fecal collection

	Soybean protein	Control	P value
	% of daily dose		
	^{65}Zn given in milk (exp. 1)		
Preliminary [1]	54.1	48.8	> 0.25
Experimental [2]	26.0	39.8	< 0.01
Adjusted experimental [3]	24.9	40.7	< 0.01

	Soybean protein	Casein	
	^{65}Zn given by capsule (exp. 2)		
Protein fed in grain [4]	27.7	30.2	> 0.25
Protein fed in milk via nipple pail [4]	18.7	34.2	< 0.05

[1] Averages of last 3 days before addition of 100 g soybean protein to milk daily for 2 calves/treatment.
[2] Averages of last 6 days of 8-day period for 2 calves/treatment.
[3] Same as footnote 2, adjusted to a common preliminary value.
[4] Averages of the last 5 days of 7- to 10-day periods for 4 calves/treatment (table 1).

4 PM in constant predetermined amounts that were consumed before the next feeding. The concentrate mixture had the following percentage composition: ground ear corn, 76.5; alfalfa meal, 5.0; cottonseed meal, 12.0; molasses, 5.0; and 0.5 each of bonemeal, dicalcium phosphate, and trace-mineralized salt.[4] Each calf received 1.5 kg of milk twice daily in nursing pails immediately before hay and grain feeding. During 2 weeks of daily oral

[4] Contained the following in g/100 g salt: Mn, 0.025; Fe, 0.100; S, 0.050; Cu, 0.033; Co, 0.015; Zn, 0.008; and I, 0.007.

dosing, the calves each received 20 μCi $^{65}ZnCl_2$ (< 1 mg Zn/mCi) in dilute HCl added to their milk twice daily. Days 4 through 6 of ^{65}Zn dosing were considered as the preliminary period (table 2). From days 7 through 14, 50 g isolated soybean protein [5] were suspended in the milk fed twice daily to each of 2 calves (table 1). The remaining 2 calves were continued with the regular ration without additional supplemental protein. Days 9 through 14 of ^{65}Zn dosing were considered as the experimental period for fecal collection. During the last 6 days of ^{65}Zn dosing, 20 μCi of ^{144}Ce (carrier-free) were added to the milk given each calf twice daily to serve as a nonabsorbed reference material for studying ^{65}Zn absorption along the digestive tracts at slaughter (16, 17). Total fecal collections were weighed, mixed, and sampled at 8:30 AM daily. Apparent net ^{65}Zn absorption was calculated as

$$100 - 100 \times \frac{\text{daily fecal } ^{65}\text{Zn excretion}}{\text{daily } ^{65}\text{Zn dose}}.$$

In the second experiment, four 93- to 140-kg calves 5 to 7 months of age were paired according to weight and age and dosed twice daily for 37 consecutive days with 20 μCi ^{65}Zn in gelatin capsules. To avoid the cottonseed meal and supplemental trace minerals in the concentrate fed in the first experiment, a ration was mixed with the following percentage composition: ground shelled yellow corn, 54; beet pulp, 39.5; molasses, 5; and 0.5 each of salt, dicalcium phosphate, and calcium carbonate. This ration was fed twice daily in constant predetermined amounts as the only feed exclusive of the protein supplement which was added separately.

Beginning the fifth day of dosing and continuing for 7 days, the 2 calves of each pair each received 50 g twice daily of either casein or isolated soybean protein mixed in the concentrate at feeding time (table 1). The protein supplements for each pair were then reversed and continued for an additional 7 days. The calves, which had been weaned from 2 to 4 months, were then offered milk in nipple pails which they readily accepted. The protein sources were suspended in milk and fed through nipple pails beginning on the second day of milk feeding. Each calf received 50 g twice daily of either casein or soybean protein. After 8 days, protein sources fed in milk to each pair were reversed and continued for an additional 10 days. During the last 9 days of ^{65}Zn dosing, each calf received 20 μCi ^{141}Ce twice daily in gelatin capsules to serve as a non-absorbed reference material. As in the first experiment, apparent net ^{65}Zn absorption was calculated from total fecal collection data.

Four hours after feeding and dosing on the last day of both experiments, all calves were stunned with a blow on the head and exsanguinated. The digestive tracts were ligated, removed, and divided into the reticulo-rumen, omasum, abomasum, three sections of equal length of small intestine, cecum, and 2 sections of large intestine. Contents of each section were weighed, mixed, and sampled.

To determine adsorption (or binding) of labeled zinc onto undigested residues, weighed ingesta samples taken throughout the digestive tract were mixed with water and centrifuged for 30 minutes at 50,000 *g*. Supernatant fractions were decanted and residues washed twice with water, each time followed by centrifugation for 30 minutes at 50,000 *g* and washings added to supernatants for each sample. The labeled zinc remaining with the washed residue was considered as bound. All samples were counted in a γ-spectrometer. When both ^{65}Zn and ^{144}Ce were present in the same sample, they were separated spectrophometrically as previously described (18). Dry matter was determined by oven drying.

Apparent net zinc absorption along the digestive tract at slaughter was calculated for each section using the equation:

$$^{65}\text{Zn absorption} = 100 - 100 \left(\frac{\%\ ^{144}\text{Ce in feed}}{\%\ ^{144}\text{Ce in ingesta}} \times \frac{\%\ ^{65}\text{Zn in ingesta}}{\%\ ^{65}\text{Zn in feed}} \right).$$

Results were expressed in relation to the previous tract section, that is, the digesta in a given organ was considered to be the feed for the next posterior organ (19), as well as in relation to the daily ^{65}Zn intake, that is, net absorption that had been accomplished up to a given organ (16). Negative absorption values indicate net secre-

[5] Assay Protein C-1, Skidmore Enterprises, Cincinnati.

tion. Statistical methods as outlined by Snedecor (20) were used.

RESULTS

During the preliminary collection period of experiment 1, apparent ^{65}Zn absorption averaged slightly although nonsignificantly ($P > 0.25$) higher for the calves later fed soybean protein (table 2). Addition of soybean protein to the milk fed these calves reduced ^{65}Zn absorption to 65% that of the controls during the last 6 days of dosing. Adjustment of treatment means by covariance to the average preliminary value for all calves reduced ^{65}Zn absorption by calves fed soybean protein to only 61% of the control average ($P < 0.01$). Apparent ^{65}Zn absorptions did not differ when soybean protein or casein were fed mixed in the grain ration during experiment 2 (table 2). However, when protein sources were fed in milk through nipple pails rather than in the concentrate mixture, net ^{65}Zn absorptions for calves fed soybean protein averaged only 55% that of calves fed casein ($P < 0.05$).

Distributions of daily administered ^{65}Zn along the digestive tract are presented in table 3. Differences between distributions of ^{65}Zn in the rumen and omasum of calves when the radioisotope was administered in milk (exp. 1) or by capsule (exp. 2) were due to point of deposition in the digestive tract (17). No differences posterior to the abomasum were noted. A negligible amount of ^{144}Ce was recovered from the rumen and omasum at slaughter in experiment 1 when ^{65}Zn and ^{144}Ce were given in milk, indicating the radioisotopes were delivered to the abomasum as expected (17). The small amount of ^{65}Zn present in the forestomach may have resulted from salivary secretion. Since the total digestive tract ^{65}Zn content at slaughter represents the accumulation of a number of daily doses, a total in excess of 100% of the daily intake when rumen contents are included is not unexpected (table 3). No effects of soy protein on ^{65}Zn distribution in the digestive tract were apparent (table 3).

Net zinc absorptions in relation to the previous section throughout the digestive tract as determined by the ^{144}Ce ratio technique are shown in table 4. This procedure indicated net ^{65}Zn secretion in the proximal small intestine as observed previously (16). Yang and Thomas (19) reported large secretions into the upper small intestine of a number of other constituents including dry matter, ash, Ca, P, Na, and water. The greatly increased value for the second relative to the first small intestine section indicated that most rapid ^{65}Zn absorption occurred in this region. No additional absorption after the cecum was apparent. Ultimate net ^{65}Zn absorptions in the lower digestive tract of calves which received ^{65}Zn in milk averaged 26 and 36%, respectively, for those fed soybean protein and their controls (table 4). Corresponding values for calves dosed by capsule and fed soy protein or casein, respectively, were 19 and 32%. This effect of soybean pro-

TABLE 3

Distributions of daily administered ^{65}Zn along the digestive tracts of calves

Section of tract	^{65}Zn given in milk (exp. 1)		^{65}Zn given by capsule (exp. 2)	
	Soybean protein	Control	Soybean protein	Casein
	% of daily dose		*% of daily dose*	
Rumen	1.9 [1]	2.1	46.8	68.8
Omasum	1.3	0.7	28.7	16.8
Abomasum	1.8	4.4	3.9	0.8
Small intestine 1	4.9	2.6	3.3	3.4
Small intestine 2	4.1	5.1	9.5	5.4
Small intestine 3	4.7	7.9	11.8	6.4
Cecum	4.5	5.6	8.2	8.8
Large intestine 1	9.1	11.7	9.6	9.1
Large intestine 2	12.6	23.3	5.9	4.2
Total	44.9	63.4	127.7	123.7

[1] Each value is the average of 2 calves/treatment.

tein in reducing apparent ^{65}Zn absorption in the lower digestive tract was significant ($P < 0.05$) in both experiments.

When ingesta from various sections of the gastrointestinal tract was extracted with water, ^{65}Zn remaining with the residue averaged 93% of the original total in samples from the rumen, omasum, cecum, and large intestine (table 5). The corresponding value for dry matter in these sections averaged 85%. Percentages of ^{65}Zn and dry matter remaining with the residue averaged 54 and 47, respectively, in samples from the abomasum and small intestine. Water-extractable ^{65}Zn and dry matter were positively related ($r = 0.75$, $P < 0.01$). Soybean protein had no apparent effect on ingesta binding of ^{65}Zn in any section but the abomasum. Both ^{65}Zn and dry matter were more completely water-extractable from abomasal ingesta when soybean protein was fed ($P < 0.05$).

DISCUSSION

Although ^{65}Zn was administered both in milk (exp. 1) and by capsule (exp. 2), this comparison is not of primary interest in the present report since no measurable zinc absorption anterior to the abomasum of cattle was detected previously (16). Thus, although ^{65}Zn administered by capsule would be expected to enter the rumen, which would act as a storage container, it would eventually pass unabsorbed into the abomasum. Rather, this study was designed to determine the effects of soybean protein on ^{65}Zn absorption in calves when the protein was delivered to the rumen by

TABLE 4

Absorption (+) or secretion (−) of ^{65}Zn along the digestive tracts of calves [1]

Section of tract	^{65}Zn given in milk (exp. 1)				^{65}Zn given by capsule (exp. 2)			
	Soybean protein		Control		Soybean protein		Casein	
	% of daily dose				*% of daily dose*			
Rumen	—	—	—	—	−78	(−78)	−67	(−67)
Omasum	—	—	—	—	−42	(15)	−11	(32)
Abomasum	48 [2]	(48) [3]	26	(26)	−8	(24)	6	(15)
Small intestine 1	−731	(−390)	−199	(−309)	−344	(−291)	−175	(−192)
Small intestine 2	−18	(58)	−34	(55)	−33	(62)	−64	(30)
Small intestine 3	28	(42)	38	(54)	25	(44)	16	(48)
Cecum	22	(42)	39	(1)	16	(−15)	36	(23)
Large intestine 1	24	(3)	35	(−6)	28	(14)	34	(−2)
Large intestine 2	31	(9)	34	(−1)	26	(−2)	36	(2)
Feces	26	(−8)	36	(2)	19	(−6)	32	(−9)

[1] Each value is the average of 2 calves/treatment.
[2] Absorption or secretion accomplished up to a particular segment of the tract.
[3] Absorption or secretion in a particular segment in relation to the previous segment.

TABLE 5

^{65}Zn and dry matter remaining with ingesta residue after washing with water

Section of tract	^{65}Zn given in milk				^{65}Zn given by capsule			
	Soybean protein		Control		Soybean protein		Casein	
	^{65}Zn	Dry matter	^{65}Zn	Dry matter	^{65}Zn	Dry matter	^{65}Zn	Dry matter
	% of total		*% of total*		*% of total*		*% of total*	
Rumen	99	94	100	95	92	71	84	69
Omasum	101	94	94	94	93	92	94	91
Abomasum	42 [1]	68 [1]	58	85	36	68	45	81
Small intestine 1	53	31	56	32	61	36	47	36
Small intestine 2	47	24	44	30	68	32	58	26
Small intestine 3	57	31	52	50	79	76	62	46
Cecum	91	86	86	84	90	82	93	82
Large intestine 1	92	87	80	82	90	82	94	85
Large intestine 2	98	92	94	90	91	82	96	82

[1] Soy protein vs. no soy protein differs significantly ($P < 0.05$) for both ^{65}Zn and dry matter.

mixing in the grain ration or to the abomasum by feeding in milk through a nipple pail.

Soybean protein effectively reduced apparent net ^{65}Zn absorption in calves only when delivered directly to the gastric stomach by feeding in milk through a nipple pail (table 2). No significant reduction in ^{65}Zn absorption was observed when soybean protein was fed in the grain ration, which ordinarily would enter the rumen. This suggests that the zinc complexing constituent in soybean protein is inactivated in the rumen. Presumably, phytate contained in the soybean protein is involved since casein did not noticeably reduce zinc absorption.

Calcium phytate in a casein-gelatin type of diet had little effect on zinc availability (7), whereas addition of sodium phytate to a chick ration depressed growth (21). This negligible effect on zinc availability of the calcium salt suggests calcium and phytic acid must react in the presence of zinc if a zinc-binding complex is formed. This can take place either before feeding (7) or in the digestive tract.

Nitrogen has been shown to enter the duodenum in large quantities in the chick (22) and calf.[6] Although the form of this nitrogen was not determined, a sizeable proportion was most likely present as protein of endogenous origin. Pekas (23) observed that secretion of ^{65}Zn into the pig digestive tract was closely associated with total protein secretion. Thus, although phytic acid decreased availability of dietary zinc in the absence of dietary protein (24), the involvement of protein of metabolic origin is not precluded. In vitro studies (25) showed that zinc binding by soybean protein was low at pH 4, increasing as pH was raised. This suggests that formation of a complex reducing zinc availability occurs posterior to the abomasum and explains the low binding of ^{65}Zn by abomasal ingesta (table 5). Since little additional zinc absorption occurred posterior to the small intestine (table 4), formation of the complex must occur anterior to the cecum to reduce zinc availability. Although net zinc secretion into the anterior small intestine was indicated (table 4), the highest rate of zinc absorption also occurred in this section of the rat digestive tract (26).

Negligible zinc absorption in sections of the tract other than the small intestine may result from the high percentage of zinc bound to undigested residues in these sections (table 5). In the small intestine, where zinc absorption appeared to be greatest, association of zinc with ingesta was least. Although only 45% of the total ^{65}Zn remained with the washed residue of gastric stomach contents, zinc absorption in this section is negligible in rats (26). In a previous report $^{65}Zn/^{144}Ce$ ratios in calves indicated some zinc absorption from this segment (16). Net ^{65}Zn absorption accomplished up to the abomasum did not differ significantly ($P > 0.05$) from that in the lower digestive tract (table 4). However, since ^{144}Ce was almost completely bound to undigested residue (17) while ^{65}Zn was only partially bound, results could have been influenced by differential passage from the abomasum.

The significance of water-extractable ^{65}Zn is uncertain since no treatment differences were apparent in the small intestine where highest ^{65}Zn absorption took place. Although the true bound fraction was probably not less than recorded by the water extraction method used, it could have been more since the measurement was made under conditions of a reduced ion concentration and shifting equilibrium toward the soluble phase.

Soybean protein, when delivered directly to the abomasum in ruminating calves, reduced zinc availability as has been observed in simple-stomach animals. Although not determined in the present report, soybean protein may pass essentially unchanged through the rumen into the abomasum in very young calves. The 19% zinc absorption obtained when soybean protein was fed in milk (table 2) should still allow adequate zinc under normal conditions. However, under conditions of borderline zinc deficiency, problems could arise. Thus, when formulating soybean protein-base milk replacers for calves, the effects on availability of zinc and possibly also certain other minerals should be considered.

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[6] Perry, S. C. 1967 Intestinal electrolyte distribution and Ca^{45} and Sr^{89} insorption in calves. Ph.D. Dissertation, University of Tennessee, Knoxville.

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Effect of Unsaturated Fatty Acid Supplements upon Mortality and Clotting Parameters in Rats Fed Thrombogenic Diets

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ABSTRACT The adhesiveness property of blood platelets has been implicated in the development of thrombosis; therefore platelet adhesiveness and other clotting parameters were studied in rats receiving the Thomas-Hartroft diet as such and as modified by supplementation with unsaturated fatty acids. Rats receiving the Thomas-Hartroft thrombogenic diet for 4 months were found to have decreased platelet adhesiveness, thromboplastin generation and platelet count. Since platelet adhesiveness is reported to increase in coronary-prone individuals, the thrombogenic diet studied does not appear to produce changes characteristic of the thrombotic tendency in man. The mortality of rats receiving the Thomas-Hartroft diet was reduced by supplementation of the diet with concentrates of linolenic, linoleic or oleic acid. However, the decreased platelet adhesiveness could be prevented by linolenic or linoleic acid, but not by oleic acid; thrombocytopenia was prevented by supplementation of the diet with linolenic acid, but linoleic and oleic acids afforded only partial protection; the decrease in thromboplastin generation could be significantly reversed only by linoleic acid. Thus, although mortality could be reduced by all 3 unsaturated fatty acids, changes in the clotting parameters studied were not necessarily affected by these treatments; and therefore, it is unlikely that these clotting parameters are involved in either the increased mortality induced by these diets or its reversal.

A number of diets which produce thrombosis and myocardial infarction (1–6), and which affect blood coagulation factors (7, 8) in rats have been described. In addition, Owren et al. (9–11) reported that individuals predisposed toward thrombosis (for example, diabetic and atherosclerotic patients) have increased platelet adhesiveness. In view of the suggested involvement of blood platelets in thrombosis a study was made of the effect of the Thomas and Hartroft thrombogenic diet on blood platelet adhesiveness, thromboplastin generation and the clotting time of recalcified whole blood. Studies were also performed with an altered thrombogenic diet which contained a lower protein level, no propylthiouracil and in which cocoa butter was substituted for dairy butter. Since at the time this investigation began, linolenic acid had been reported to reduce platelet adhesiveness in individuals with high values (9), the effect of this and other unsaturated fatty acids upon this clotting parameter was examined.

EXPERIMENTAL PROCEDURE

Diets. Four diets were prepared, two thrombogenic, one high in fat but minus the "thrombogenic" factors, and one control diet. One thrombogenic diet was formulated as described by Thomas and Hartroft (1). The second was formulated by substituting cocoa butter for dairy butter, omitting the propylthiouracil, and using a lower protein level (11%). The latter diet was prepared because of reports that use of cocoa butter eliminated the need for propylthiouracil (8), and that a lower protein level increased the incidence of thrombosis (6). The high fat diet contained dairy butter but lacked the "thrombogenic" factors (cholesterol, sodium cholate, choline chloride and propylthiouracil). The compositions of the basal diets are summarized in table 1.

Diet treatment. Rats were divided initially into 4 groups: 1) Thomas and Hartroft thrombogenic diet, 2) diet 1 with cocoa butter substituted for dairy butter, and containing 11% protein and no propylthiouracil, 3) diet 1 minus the "thrombogenic" factors, and 4) purified control diet. After a basal period (2 to 4 months) the animals in groups 1, 2 and 4 were divided and the subgroups given supplements of various fatty acid concentrates. The length

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TABLE 1

Composition of basal diets

	Basal thrombogenic (Hartroft (1))	Cocoa butter thrombogenic	High fat	Control
Cornstarch [1]	—	—	—	71.0
Casein [2]	20.0	11.0	20.0	18.0
Cottonseed oil [3]	—	—	—	5.0
Sucrose	20.5	20.5	20.5	—
Non-nutritive fiber [4]	6.0	25.8	6.0	—
Vitamin mix [5]	2.0	2.0	2.0	2.0
Salt mix [6]	4.0	4.0	4.0	4.0
Choline chloride [4]	0.2	0.2	0.2	—
Sodium cholate [4]	2.0	2.0	2.0	—
Propylthiouracil [4]	0.3	—	0.3	—
Cholesterol [4]	5.0	5.0	5.0	—
Dairy butter	40.0	—	40.0	—
Cocoa butter [7]	—	40.0	—	—

[1] Amidex, Corn Products Company, Argo, Illinois.
[2] ANRC reference protein, Sheffield Chemical, Norwich, New York.
[3] Wesson Oil, Wesson Sales Company, Fullerton, California.
[4] General Biochemicals, Chagrin Falls, Ohio.
[5] Each kilogram of vitamin mixture contained the following triturated in dextrose: (in grams) vitamin A conc (30,000 IU/g), 30; vitamin D conc (30,000 IU/g), 3.33; α-tocopheryl acetate, 5.0; ascorbic acid, 45.0; inositol, 5.0; 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; biotin, 0.2; and folic acid, 0.09.
[6] Salt composition was a modification of that described by Wesson (12). The composition in percent by weight is as follows: sodium chloride, 10.5; potassium chloride, 12.0; potassium dihydrogen phosphate, 31.0; calcium phosphate [$Ca_{10}(OH)_2(PO_4)_6$], 14.9; calcium carbonate, 21.0; magnesium sulfate, 9.0; ferric chloride ($6H_2O$), 1.72; manganous sulfate ($5H_2O$), 0.039; sodium fluoride, 0.057; and potassium iodide, 0.005.
[7] Mutchler Chemical Company, New York.

of the basal period was chosen on the basis of the report that rats fed these thrombogenic diets die with massive thrombi after 2 months (3). The control animals, for example, were divided into 2 groups, one was continued with the control diet, and the other received the same diet to which a linolenic acid concentrate [1] was added at a level of 5% (w/w). The animals fed the Thomas and Hartroft diet were divided into the following 4 groups: 1) continued with the basal thrombogenic diet, 2) maintained with the basal diet supplemented with 5% linolenic acid concentrate, 3) basal diet supplemented with 5% linoleic acid concentrate, and 4) basal diet supplement with 5% oleic acid concentrate.[1] These supplements contained 86 to 88% unsaturated fatty acids; the saturated fatty acid components were chiefly myristic, palmitic and stearic acids. The amounts of the various unsaturated fatty acids added, based on gas chromatographic analyses are shown in table 2. In comparing the linolenic- and linoleic acid-supplemented groups, the factor controlled was the *total* amount of polyunsaturated acids; the relative amounts of the 2 acids varied. Additional experiments involved a study of the addition of triolein and greater quantities of butter to the basal Hartroft diet.

Blood collection. Except where stated, silicone-coated glassware was used exclusively in collection and assays. Blood (1.8 ml) was collected by intracardiac puncture under light anesthesia [2] into 0.1 M sodium citrate-citric acid buffer (3:2), pH 4.6, 1 part anticoagulant to 9 parts of blood. From a total of 2.0 ml, 0.1 ml was taken for the recalcification time determination, 0.4 ml was set aside to be centrifuged for the thromboplastin generation determination and the remainder was used for assay of platelet adhesiveness. Blood was withdrawn for the various tests at approximately one-month intervals.

Platelet adhesiveness determination. A glass tube (5.0 mm ID) was packed with 5.0 g of uncoated glass beads [3] (0.5-mm average diameter). Approximately 1.5 ml of citrated blood was added (15 minutes after withdrawal) and sufficient pressure applied to the head of the column so that passage time was 35 to 40 seconds. Under

[1] Linolenic, linoleic and oleic acid concentrates obtained from Nutritional Biochemicals Corporation, Cleveland.
[2] Fluoromar (trifluoroethyl vinyl ether), Ohio Chemand Surgical Company, Chicago.
[3] Dragon-Werk, Bayreuth, West Germany.

TABLE 2

Fatty acids provided by dietary supplements

Supplement [2]	Fatty acids added to diet [1]			
	Linolenic	Linoleic	Oleic	Palmitoleic
	g/100 g diet			
Linolenic acid conc [3]	2.2	0.9	1.3	—
Linoleic acid conc [3]	—	3.1	1.2	0.1
Oleic acid conc [3]	0.04	0.14	3.1	0.9

[1] Data based on chromatographic analyses of the concentrates.
[2] Each fatty acid concentrate was added to the Hartroft thrombogenic diet at a level of 5% w/w.
[3] Nutritional Biochemicals Corporation, Cleveland.

these conditions the retention of platelets become relatively insensitive to slight variations in flow time. A small amount of the column eluate was collected and 0.1 ml added to 4.9 ml of the citrate anticoagulant. The red cells were allowed to settle for one hour before platelet counting was begun. A standard hemocytometer was filled and placed in a covered petri dish (bottom covered with moist filter paper) for 15 minutes. Counting was performed using conventional optics and the standard procedure for red blood cells. Counts obtained in this manner were compared with those obtained with the same specimen of blood which had not been subjected to the column treatment and the percentage decrease determined. Adhesiveness values with this procedure approximated 50% using blood from rats fed a normal diet.

Determination of thromboplastin generation. Citrated blood was centriufged [4] 30 minutes at maximum speed. The platelet-poor plasma was assayed for generation of thromboplastic activity [5] essentially as described by Silver et al. (13). A platelet substitute was not used as sufficient generation of activity was obtained without it.

Recalcification time of whole blood. Citrated blood (0.1 ml) was placed in the cup of a clot timer. To this were added 0.1 ml of imidazole buffer, pH 7.4 (13) and 0.1 ml of 0.025 M $CaCl_2$, and the clotting time was determined. All clotting tests (including thromboplastin generation) were determined with a mechanical clot timer.[6]

RESULTS

Mortality. Data from 2 experiments showing the effect of various dietary supplements on the death rate of rats fed the Hartroft thrombogenic diet are tabulated in tables 3 and 4. Supplementation with each of the fatty acid concentrates (after a basal period) reduced the number of deaths compared with that of the group of rats continued with the Hartroft diet without supplementation. Summation of the chi square values of the 2 experiments show a significant effect for linolenic ($P < 0.025$) and oleic acids ($P < 0.05$). The effect of linoleic acid was not significant although a definite trend was indicated ($P < 0.1$). The results suggest that no unique effect on mortality reduction can be ascribed to linolenic acid. No deaths occurred in the group maintained

TABLE 3

Effect of fatty acid supplementation on the mortality of rats fed a thrombogenic diet

Diet group	No. of animals (initial)	No. deaths
Control	10	0
Thrombogenic	13	7
Thrombogenic (2 months) [1] + linolenic acid	10	1
Thrombogenic (2 months) + linoleic acid	10	2
Thrombogenic (2 months) + oleic acid	10	1
High fat, no thrombogenic factors	10	0
High fat (2 months) + linolenic acid	10	0
Control (2 months) + linolenic acid	10	0

[1] Animals maintained with the thrombogenic diet (Thomas and Hartroft (1)) 2 months and then subdivided into the various supplemented groups. Animals fed the high fat and control diets were treated in similar fashion. These data were compiled 46 days after subgrouping.

[4] International Clinical Centrifuge, Model CL.
[5] Diagnostic plasma was obtained from Warner-Chilcott Laboratories, Morris Plains, New Jersey.
[6] Mechrolab Inc. (Model 202), Mountain View, California.

TABLE 4

Effect of various supplements on the mortality of rats fed the Hartroft thrombogenic diet

Diet supplement [1]	No. of animals (initial)	No. deaths
None	10	5
Linolenic acid [2]	9	2
Oleic acid [3]	8	2
Triolein [4]	8	3
5% additional butter	8	6

[1] Animals were maintained with the basic thrombogenic diet 4 months, then subgrouped for supplementation. Mortality tabulations compiled 7 weeks after subgrouping.
[2] Linolenic acid, 2.2%; linoleic, 0.9%; oleic, 1.3%—based on total diet.
[3] Oleic acid, 3.1%; palmitoleic, 0.9%.
[4] Oleic acid (glyceride form), 3.1%; palmitoleic, 0.3%. Nutritional Biochemicals Corporation, Cleveland.

with the high fat diet minus the "thrombogenic" factors, a result similar to that reported by Naimi et al. (14).

Animals fed diets supplemented with 5% additional butter after the basal period with the Hartroft diet exhibited high mortality (table 4), indicating that certain butter components plus the "thrombogenic" components constitute a particularly lethal combination. The data in table 4 were obtained with animals from which blood was not drawn tc avoid the effect of trauma introduced by blood sampling.

Histologic examination of the hearts and kidneys of the animals that died during the experiment revealed thrombus-like masses in the cardiac chambers and occasionally in the coronary vessels. It cannot be concluded with certainty that these deposits represent masses formed before death or are merely postmortem clots—a reservation shared by other investigators (15).

Platelet adhesiveness. Significantly lower platelet adhesiveness values were obtained with animals fed the Hartroft thrombogenic diet (table 5). The adhesiveness values of animals receiving the thrombogenic diet showed a further decrease during the 2-month supplemental period, a change also observed in animals fed the control and high fat diets. The decline in platelet adhesiveness in animals fed the Hartroft diet was halted by addition of linolenic acid or linoleic acid to the diet. However, addition of the monounsaturated oleic acid resulted in further lowering of platelet adhesiveness. A decrease in platelet adhesiveness was also observed in a similar experiment in which cocoa butter was substituted for dairy butter and propylthiouracil omitted.

Platelet count. The platelet count was significantly higher in animals fed the Hartroft diet for 2 months than in those fed the control diet (table 6), which is consistent with results reported by Davidson et al. (7). However, the animals became definitely thrombocytopenic when maintained with the Hartroft diet for 4

TABLE 5

Effect of diet supplements on rat platelet adhesiveness

Diet group	Adhesiveness	
	Basal period [1]	After supplemental period
	%	%
Control, no supplement	51±2 [2](21)	41±4 (9)
Control (linolenic acid) supplement at 2 months	51±2 [2]	36±5 (12)
Thrombogenic, no supplement	40±2 [3](41) [1]	31±6 (6)
Thrombogenic (linolenic acid supplement at 2 months)	40±2 [3]	48±8 (7)
Thrombogenic (linoleic acid supplement at 2 months)	40±2 [3]	40±6 (8)
Thrombogenic (oleic acid supplement at 2 months)	40±2 [3]	17±5 [4](7)
High fat,[5] no supplement	53±3 (20) [1]	36±6 (9)
High fat (linolenic acid supplement at 2 months)	53±3	40±7 (9)

[1] Animals subgrouped (on basis of adhesiveness) after 2 months (basal period) and then given the supplemented diets for 2 months.
[2] SE of mean; numbers in parentheses indicate number of animals.
[3] Significantly lower than control or high fat group, $P < 0.05$.
[4] Significantly lower than the thrombogenic group before supplementation, $P < 0.025$.
[5] High fat minus the "thrombogenic" factors (cholesterol, cholate, choline, propylthiouracil).

TABLE 6

Effect of diet supplements on platelet counts in rat blood

Diet group	Basal period [1]	After supplemental period
	count/mm³ × 10³	
Control, no supplement	850 ± 31 [2](23)	827 ± 40 (9)
Control linolenic acid supplement at 2 months	850 ± 31 [2]	776 ± 37 (12)
Thrombogenic (Hartroft), no supplement	998 ± 45 [3](45)	522 ± 126 [4](6)
Thrombogenic (linolenic acid supplement at 2 months)	998 ± 45 [3]	1000 ± 75 [5] (7)
Thrombogenic (linoleic acid supplement at 2 months)	998 ± 45 [3]	807 ± 51 (8)
Thrombogenic (oleic acid supplement at 2 months)	998 ± 45 [3]	714 ± 67 (7)
High fat,[6] no supplement	880 ± 31 (20)	743 ± 18 (9)
High fat (linolenic acid supplement at 2 months)	880 ± 31	775 ± 37

[1] Basal period 2 months, before subgrouping.
[2] SE of mean; numbers in parentheses indicate number of animals.
[3] Significantly higher than control group, $P < 0.05$.
[4] Significantly lower than control group, $P < 0.05$; significantly lower than thrombogenic + linolenic group, $P < 0.01$; significantly lower than thrombogenic + linoleic group, $P < 0.05$.
[5] Significantly higher than control group, $P < 0.05$; significantly higher than thrombogenic + linolenic group, $P < 0.05$; significantly higher than thrombogenic + oleic group, $P < 0.025$.
[6] High fat minus the "thrombogenic" factors (cholesterol, cholate, choline, propylthiouracil).

months. A similar decline in platelet count occurred in a subsequent experiment, although 2 months earlier, indicating that this condition is reproducible.

On addition of linolenic acid to the Hartroft diet (after the 2-month basal period), the thrombocytopenia which occurred on continuation of the basal diet was not observed; addition of linoleic or oleic acid to the diet afforded less protection. The decline in platelet count in the high fat diet group (minus the "thrombogenic" factors) during the third and fourth months is at some variance with the data reported by Naimi et al. (14) who did not observe a decrease until after one year. However, these investigators included choline chloride in the diet, and a high fat diet may increase the requirement for this nutrient (16). In animals fed the high fat diet supplemented with linolenic acid the decrease in platelet count (table 6) was somewhat smaller and not statistically significant.

Thromboplastin generation. Significantly greater thromboplastin generation was observed after one month with animals fed the Hartroft and cocoa butter diets than with those on the control diet (fig. 1). After 2 months a reversal occurred, particularly in the cocoa butter group.

In table 7 data are presented showing the effect of fatty acid supplementation (during the third and fourth months) of the Hartroft thrombogenic diet and the high fat diet. Addition of linolenic and oleic acids accelerated thromboplastin generation (decreased clotting time) although not significantly. Linoleic acid addition significantly increased generation after 6 minutes' incubation of the generation mixture. No change was observed following addition of linolenic acid to the diet of the high fat group. The latter group showed "normal" thromboplastin generation; and similarly such a regimen has been reported by others to produce normal activity (14).

Clotting time of recalcified whole blood. The effect on clotting time of fatty acids added to the various diets (after the basal period) is shown in table 8. None of the supplements produced significant changes.

DISCUSSION

Mortality of rats fed the Hartroft thrombogenic diet was significantly reduced when the diet was supplemented with either linolenic or oleic acid; the results with linoleic acid, though not significant, showed a similar trend.

Animals fed the thrombogenic diets exhibited decreased platelet adhesiveness values after 2 months, contrary to what might be expected since predisposition toward human coronary thrombosis is reportedly associated with an increase in the platelet adhesiveness value (9). Conceivably the

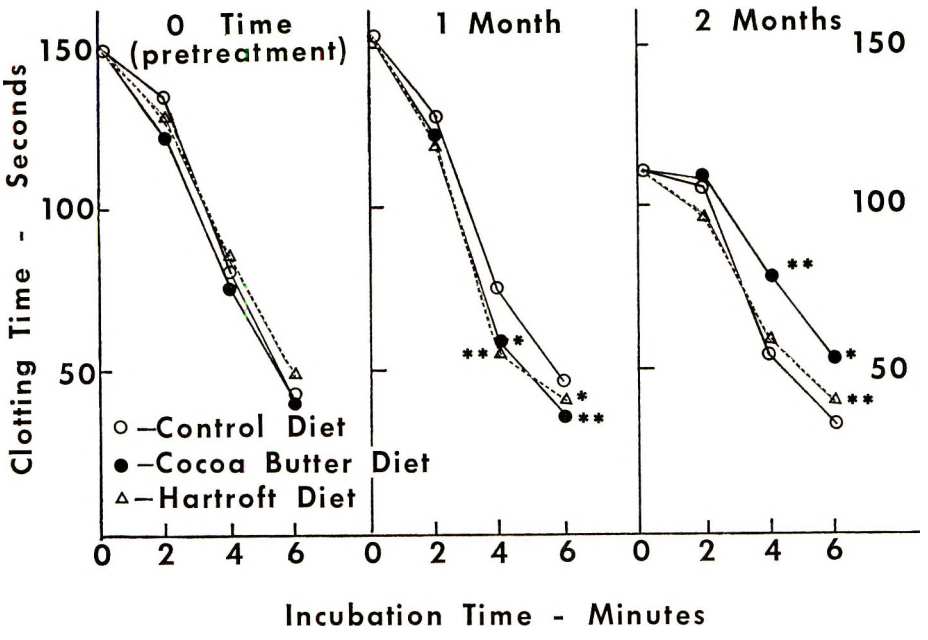

Fig. 1 Effect of diet on rat thromboplastin generation. Significantly different from control, * $P < 0.01$; ** $P < 0.001$.

TABLE 7

Effect of diet on thromboplastin generation in rat plasma

Diet group	Generation mixture incubation time		
	2 min	4 min	6 min
	Test plasma clotting time		
	seconds		
Thrombogenic, Hartroft (4 months) (6) [1]	143 ± 8 [2]	101 ± 22	85 ± 21
Thrombogenic (2 months) + linolenic acid (2 months) (9)	128 ± 8	71 ± 7	51 ± 5
Thrombogenic (2 months) + linoleic acid (2 months) (8)	123 ± 7	66 ± 8	45 ± 4 [3]
Thrombogenic (2 months) + oleic acid (2 months) (7)	148 ± 7	92 ± 14	61 ± 9
High fat, no thrombogenic factors (4 months)(8)	130 ± 10	68 ± 10	38 ± 3
High fat (2 months) + linolenic acid (2 months) (9)	127 ± 14	71 ± 10	39 ± 3

[1] Numbers in parentheses indicate number of animals/group.
[2] SE of mean.
[3] Significantly lower than 6 minutes thrombogenic group, $P < 0.05$.

more adhesive platelets were removed from circulation and those remaining had lower adhesiveness values. However, in work reported here and by others (7) the platelet count in the thrombogenic group was higher than the control, indicating that this is not the case.

The thromboplastin generation values which had increased at one month with both the cocoa butter and Hartroft diets were lower than those of the controls after 2 months. Possibly this was caused by the general deterioration in the condition of the animals. Davidson et al. (7) decribed a generation defect in rats fed the Thomas-Hartroft diet; later this report was retracted (8). Also, since the data of Davidson et al. were obtained by pooling values obtained from animals killed over a period of 14 to 90 days (7), their results are

TABLE 8

Effect of diet on rat whole blood recalcification time

Diet group	Clotting time
	seconds
Thrombogenic, Hartroft (4 months) (6) [1]	77±8 [2]
Thrombogenic (2 months)+ linolenic acid (2 months) (8)	65±4
Thrombogenic (2 months)+ linoleic acid (2 months) (8)	66±5
Thrombogenic (2 months) + oleic acid (2 months) (7)	76±5
High fat, no thrombogenic factors (4 months) (9)	72±4
High fat (2 months)+linolenic acid (2 months) (9)	74±4

[1] Numbers in parentheses indicate number of animals/group.
[2] SE of mean.

difficult to compare with those obtained in the present studies.

The reduction in mortality produced by supplementation of the Thomas-Hartroft diet was not mediated through a reduction in platelet adhesiveness nor was the decrease correlated with a reduction in thromboplastin generation. This leaves open the question as to what mechanism is operative in producing these effects. In table 2, it is shown that the fatty acid supplements used in this study, although predominately of one type, contained mixtures of fatty acids. Thus, 5% supplementation with the linolenic acid concentrate added 2.2 g of this acid per 100 g of diet, but it also added 0.9 g of linoleic acid (the concentrate contained 17.9% of the latter). Assuming a consumption of 10 to 20 g of diet per day, the additional intake of linoleic acid per day would be 90 to 180 mg, approximately a 100% increase over the amount consumed with the nonsupplemented diet. Although the linoleic acid consumption on the nonsupplemented diet (80–160 mg per day) would normally be considered adequate, a regimen high in saturated fat may require a higher level of polyunsaturated fatty acids (16). Thus, supplementation with the linolenic acid concentrate may have corrected a unique deficiency in linoleic acid. The same reasoning could apply to the diet supplemented with linoleic acid concentrate, since a total of 3.1 g of this essential fatty acid per 100 g of diet was added from this source. However, it should be noted that the linoleic acid supplement supplied no linolenic acid.

The oleic acid concentrate also contained a small quantity of linoleic acid so that this concentrate also increased the linoleic intake 14 to 28 mg per day and it is conjecture whether this modest increase — approximately 20% would be an important factor in reducing the death rate. The 30% increase in oleic acid resulting from supplementation with the oleic concentrate (from 10.8 to 13.9 g/100 g of diet) could exert a protective effect although this hardly seems likely.

Consideration was given to the possibility that the form in which the fatty acid supplement was added is a critical factor. The fatty acids in the thrombogenic diets exist predominantly in glyceride form while in the supplements they were added as free acids. The results obtained with the group supplemented with triolein (table 4) are equivocal, and a larger number of animals are needed to demonstrate that more protection is afforded by fatty acids than by triglycerides. However, addition of 5% additional butter to the thrombogenic diet appeared to increase mortality. While other factors may be involved, the amount of oleic acid added with 5% additional butter was nearly the same as with the oleic acid supplement; the difference is that the oleic acid added in the butter would be in the glyceride form.

It is possible that some minor component in these supplements is responsible for the decreased mortality. However, it was noted above that the minor components consist mainly of saturated fatty acids. Two of them, myristic and palmitic acids are reportedly major dietary inducers of hypercholesterolemia (17); thus any effect of these components would be expected to increase rather than reduce mortality. With respect to the possible effect of vitamins in the supplement concentrates, Renaud and Allard report that vitamin addition aggravated the pathological changes produced by a laboratory ration diet which had been supplemented with butter fat, cholesterol and sodium cholate (5). It is conceivable that the relatively high content of total fat and of unsaturated fatty acid (in the supplemented diets)

would increase the requirement for α-tocopherol. However the daily intake was 2 mg per day (based on food consumption of 10 g); 0.75 mg per day has been reported as adequate in male rats receiving a diet containing 22% lard and 2% cod liver oil (18).

Another mechanism by which the fatty acids could reduce the death rate is through prevention of thrombocytopenia. It is possible that the decline in platelet count in the nonsupplemented group resulted in the more adhesive platelets adhering to the vessel wall causing small thrombi and being selectively removed. The concurrent release of ADP from the adherent platelets may induce additional aggregation and a further decline in platelet count. The fatty acid supplements thus could act by: 1) improving the integrity of the vessel wall and thereby decreasing the initial adherence of the platelets, 2) by counteracting the ADP induced platelet aggregation, or 3) by altering the surface characteristics of the platelets themselves. The effect of linolenic acid in protecting animals fed the thrombogenic diet against thrombocytopenia appears to be unique since supplementation with linoleic or oleic acid concentrates afforded only partial protection.

A significant result of this study concerns the effect of the thrombogenic diets on platelet adhesiveness. Although, as suggested above, the decrease in this parameter may be more apparent than real because of a possible selective removal of the more adhesive platelets, it must be noted that at no time was an increase in the adhesiveness value observed. Since predisposition toward thrombosis in man has been found to involve an increase in this property, it may be concluded that the condition induced by these diets does not mimic that observed clinically in humans. In this respect other investigators have expressed reservations concerning the correspondence of the thrombus masses observed in these animals to those found in man (15).

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