Effect of Protein Depletion on Urinary Nitrogen Excretion in Undernourished Subjects

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ABSTRACT The extent of labile protein stores and their response to protein feeding was studied in undernourished adult subjects. Urinary nitrogen excretion following 3 levels of protein intake (62, 82 and 96 g/day), each protein period alternated with a protein-free diet, was studied in 4 apparently healthy but undernourished young men. Urinary nitrogen with a protein-free diet decreased to a nearly steady value within 2 to 3 days. The initial decrease was small, however, indicating poor labile protein stores in these subjects. With diets containing protein considerable nitrogen was retained. The retained nitrogen, however, was not excreted when the subjects were changed over from protein-containing diets to protein-free diets. Endogenous urinary nitrogen excretion in these subjects was not different from that of normal subjects.

There have been several studies of the effect of variation of dietary protein intake on the urinary nitrogen excretion (1–3). These studies indicate that there are apparently some components in the body which are comparatively more sensitive to changes in protein intake than others. One of these, body protein, which is particularly sensitive to changes in protein intake, has been termed the labile protein store. The presence of labile protein stores in different species of animals has been inferred from urinary nitrogen excretion immediately following consumption of a protein-free diet (1, 2, 4). Whenever the level of protein intake is altered, there is often a time lag in the adjustment of the body to a new level of protein, which is reflected in a corresponding variation in the excretion of nitrogen. Several investigators have observed (5–11) that in human subjects, a protein-free diet following a normal protein intake causes urinary nitrogen to decrease rapidly at first and then slowly to reach a fairly steady level which has been termed the endogenous level. Extra nitrogen lost in the first few days over and above the endogenous level has been considered to correspond to the labile protein store. According to Martin and Robison (7), this labile store represents about 3% of total body proteins, and under the most favorable circumstances does not exceed 5% (3).

The labile protein stores are drawn upon during periods of stress (12). Although the actual physiological significance of labile protein stores is not fully understood, it is agreed that adequate stores of labile proteins are consistent with good protein nutritional status. In population groups habitually subsisting on marginal intakes of protein, labile protein stores may be expected to be low. In any consideration of protein requirement, in addition to the protein requirement for maintaining N balance protein required to maintain labile protein stores merits attention.

The present paper describes studies of nitrogen balance in apparently healthy but undernourished subjects eating protein-free diets, and the effects of fluctuation in the levels of protein intake on the pattern of urinary nitrogen excretion.

MATERIALS AND METHODS

Subjects. Four apparently healthy young men belonging to the low income group were the subjects of this study. They were kept in a metabolic laboratory during the entire period of the experiment. The basal metabolic rates of these subjects were determined initially. Details of the subjects are shown in table 1.

Diet: The subjects were given a protein-free diet alternating with a high pro-

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TABLE 1

Description of the subjects

Subject	Age	Weight	Height	Surface area	Basal energy
	years	*kg*	*cm*	cm^2	*kcal/day*
JB	25	44.8	161.4	1.43	1292
MS	26	45.5	162.6	1.44	1076
JS	31	48.4	166.0	1.50	1272
NKS	28	45.4	163.1	1.46	1250

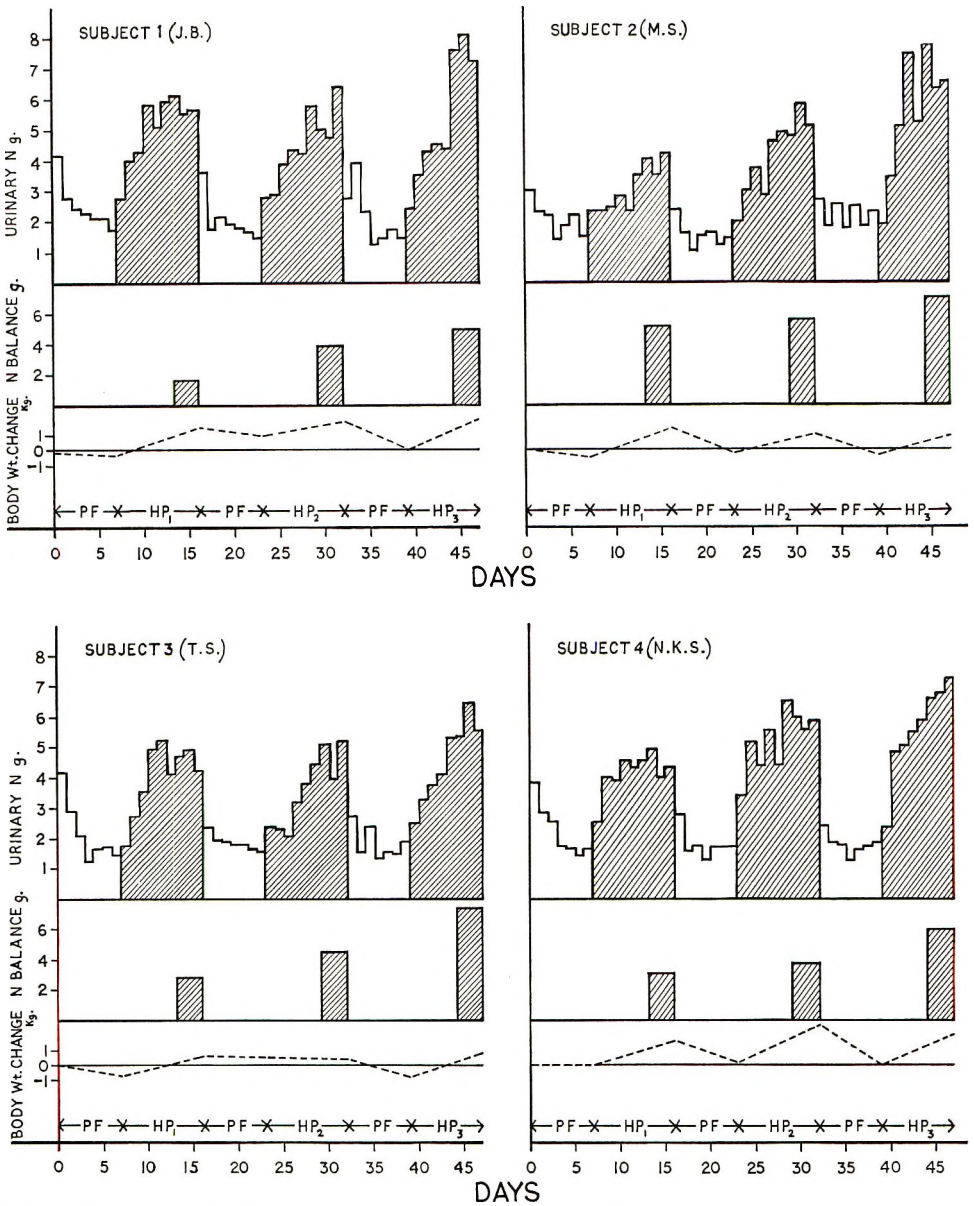

Fig. 1 Nitrogen metabolism of men eating protein-free (PF) and high protein diets (HP_1, HP_2 and HP_3).

tein diet. Each subject was studied for 3 separate periods with the protein-free diet, with each period followed by a high protein diet. Daily protein intake during the 3 protein periods was 62, 82 and 96 g, respectively. The duration of each protein-free dietary period was 7 days and that of the high protein period was 9 days. The sequence of feeding the different diets is shown in figure 1. The protein-free diet included sago, cornstarch and vegetables low in nitrogen and did not contain any protein food. In formulating the practical protein-free diet, however, small quantities of nitrogen from sago and vegetables could not be avoided entirely. Protein in the high protein diets was derived mainly from rice, wheat, red gram dhal (*Cajanus cajan*) and milk. The composition of the diets is shown in table 2.

Urine was collected daily on glacial acetic acid and toluene during the entire period of the study. Feces were collected during the last 3 days of each period. Body weights were taken at the beginning and at the end of each dietary period. Total nitrogen was estimated in urine and feces by the macro-Kjeldahl method.

RESULTS

Average daily urinary nitrogen output, nitrogen balance with the high protein diets and body weight changes during the different dietary periods are shown in figure 1. Body weight changes during the different periods were 1 to 2 kg. Body weight lost during the protein-free regimen was largely made up during subsequent protein feeding.

Urinary excretion of nitrogen decreased rapidly in all the subjects to about 2 g within 2 to 3 days of the protein-free regimen and remained steady or decreased rather slowly thereafter. The magnitude of the initial decrease in urinary nitrogen excretion with the protein-free regimen, however, was quite small in these subjects, unlike the sharp decrease in normal subjects eating protein-free diets that was reported by Martin and Robison (7). Moreover, the 2 distinct phases of urinary nitrogen excretion reported by other workers were not strictly in existence in all the subjects. This indicated that the significant initial response to the protein-free diet reported in well-fed subjects was absent or minimal in our subjects. When our subjects were fed a high protein diet following the protein-free diet, urinary nitrogen excretion increased rapidly and tended to reach a plateau. Even after 9 days of protein feeding the subjects had not attained N equilibrium.

Average daily urinary nitrogen excretion during the last 3 days of the protein-free diet is shown in table 3. Following the usual convention, these values were taken to represent endogenous excretion

TABLE 2

Composition of experimental diets

	Protein-free diet	Protein diets		
		1	2	3
	g	*g*	*g*	*g*
Sago	255	—	—	—
Rice	—	300	400	450
Wheat flour	—	100	133	170
Cornstarch	210	—	—	—
Red gram dhal	—	50	66	100
Skim milk powder	—	20	27	40
Sugar	90	60	52	28
Oil	90	40	10	20
Calabash cucumber	240	—	—	—
Ridge gourd	250	—	—	—
French beans	—	200	250	275
Potato	—	200	300	—
Cauliflower	—	100	100	100
Onions	—	50	50	25
Protein, g	3.4	62.4	81.6	95.7
Kilocalories	3007	3004	3006	3022

TABLE 3

Nitrogen output and body nitrogen loss of men eating a protein-free diet

Subject	Avg daily N output during last 3 days of protein-free diet period						Body nitrogen loss [1] during protein depletion period		
	Period 1		Period 2		Period 3		Period 1	Period 2	Period 3
	Urinary	Fecal	Urinary	Fecal	Urinary	Fecal			
	g	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*
JB	1.94	0.91	1.61	1.39	1.57	1.36	3.82	2.86	4.02
MS	1.81	0.85	1.39	0.99	2.18	1.02	2.73	0.81	0.55
JS	1.62	0.89	1.66	1.03	1.66	1.37	3.83	1.42	1.47
NKS	1.57	1.00	1.70	0.91	1.75	1.13	4.60	0.78	0.34

[1] Urinary nitrogen excreted over and above the endogenous minimum.

of nitrogen. Endogenous urinary nitrogen values with the protein-free diet were almost the same in the 3 protein-free periods irrespective of the protein intake preceding each period and ranged from 1.5 to 2.0 g/day. The endogenous urinary N output per basal kilocalorie ranged from 1.30 to 1.65 mg, the average for the 4 subjects being 1.4 mg/basal kcal. This value is not different from values for normal subjects reported by other workers (10, 13–15). Fecal N excretion with the protein-free diet ranged from 0.89 to 1.37, the average value being 1.07 g/day. This value is not very different from the values reported by Murlin et al. (13), and Mueller and Cox (11) in two of his subjects, but it is higher than values reported by Bricker and Smith (14) and Hawley et al. (15).

Extra urinary nitrogen lost over and above the endogenous minimum during the protein depletion period after the subjects were transferred to the protein-free diet is also shown in table 3. Body nitrogen loss during this period ranged from 0.34 to 4.6 g which was less than 1% of body protein in the 4 subjects. This loss was highest in the first protein-free diet period and decreased in the subsequent 2 protein-free diet periods in three of the four subjects. In subject JB nitrogen loss did not differ greatly in the 3 protein-free diet periods. Short periods of high protein feeding prior to use of the protein-free diet did not increase the loss of body proteins observed with protein-free diet.

The average values for daily nitrogen retention during the last 3 days of the 3 high protein regimens are shown in figure 1. Total nitrogen retained during the entire period of 9 days would be much larger. Nitrogen retained even during the last 3 days of the diet was considerable, the higher the protein level in the diet the more nitrogen retained. These results suggested that nitrogen retained during the high protein regimen was substantially higher than nitrogen lost during the preceding protein-free dietary period.

DISCUSSION

The initial rapid urinary nitrogen loss following the protein-free diet has been considered to represent loss of labile pro-

tein stores of the body. The decrease in urinary nitrogen excretion following a protein-free diet in well-nourished individuals consists of 2 phases, one rapid phase when the urinary nitrogen excretion decreases to a low value within a few days and the other a slow phase when the nitrogen excretion decreases rather slowly until it reaches a steady state. This pattern has been observed both in human subjects and experimental animals (1-4). Nitrogen loss in the first phase is considered to represent loss of labile protein stores which may be 3 to 5% of body proteins. Nitrogen loss in the subsequent slow phase may represent loss of more stable body proteins. In the present study which concerned relatively undernourished subjects, however, the pattern of urinary nitrogen loss with a protein-free diet appeared to correspond mainly with the slow phase, the rapid phase being absent or minimal. This indicated that highly labile protein stores such as those observed in normal persons were minimal or absent in these subjects. This conclusion is supported further by the fact that the calculated body nitrogen loss in our subjects during the protein-free regimen was less than 1.0% of body nitrogen compared with a loss of 3 to 5% of body nitrogen reported for well-nourished subjects. Relative tissue depletion in our subjects is also supported by the tendency of the subjects to retain large quantities of nitrogen when they were maintained with a high protein diet following a protein-free diet. The subjects of this study normally subsisted on marginal protein intakes and it may be argued that they could not have built up adequate labile protein stores. It appears from this study that the pattern of body protein loss with a protein-free diet depends upon the state of protein stores in the body. The response to a protein-free diet can be expected to be poor, as in our subjects, if only less labile proteins are present in the body since this component of protein could be expected to resist depletion. The significance of the poor response of our subjects to a protein-free diet in terms of their proteins stores needs further study.

Loss of urinary nitrogen during the 3 protein-free periods apparently was not influenced by the preceding short periods of high protein feeding which resulted in a large positive nitrogen balance. Nitrogen retained with high protein feeding was obviously not excreted when these subjects were transferred to a protein-free diet, a deviation from normal response. This can be interpreted to mean that these subjects had extensively depleted protein stores and that nitrogen retained by them was presumably repleting preferentially less dispensable body protein stores and not highly labile protein stores. Perhaps a prolonged period of high protein feeding to these subjects to the point where their body protein stores were completely filled would be necessary before they could respond to a protein-free diet as well as well-nourished subjects.

One of the points of practical significance in the present study concerns the protein requirement of undernourished subjects. Since endogenous nitrogen excretion in our subjects was not different from that reported for normal subjects, the minimal protein requirement of these subjects necessary to take care of the obligatory loss is apparently not different from that of normal well-nourished subjects. Since these subjects usually have very poor stores of reserve proteins, a protein allowance to cover only the minimal needs would never provide an opportunity for them to replete their body protein stores fully. Therefore, the basis for computation of the protein requirement in such subjects would obviously differ from that for normal subjects. Although the significance of labile protein stores in normal subjects is not understood clearly, its beneficial effect during times of stress may be important.

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Effect of Selenium and Vitamin E on the Regeneration of Rat Liver

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ABSTRACT Because previous studies have shown the hepatoprotective effect of selenium (a constituent of Factor 3), the effect of selenium on the hepatic regeneration of albino rats was studied. The experiments used 125 animals, divided into 4 groups. The control group was maintained with a semipurified diet. The 3 experimental groups received the same basal diet which was supplemented with Na_2SeO_3, vitamin E or Na_2SeO_3 and vitamin E together. The weight increase of the hepatic regenerate was observed, as well as its microscopic appearance and the percentage of the different forms of cell division. It was found that selenium caused an increase in weight of the residual liver, favoring selectively the mitotic processes, and, combined with vitamin E, it stimulated all forms of cell division in the liver. The physiological dose used for stimulation of liver regeneration was about 1 μg/100 g body weight. When selenium was given in larger doses, it produced certain vascular lesions, appearing in the form of parenchymatous hepatic microhemorrhages.

The experimental investigations of the last 15 years have shown the importance of selenium (Se) as a basic element of nutrition and as one of the constituents of Factor 3, which is being widely discussed in the current literature. According to Schwarz et al. (1),[2] both tocopherol and selenium participate in oxidation-reduction reactions. Since the metabolic lesions found in the prenecrotic, latent phase of dietary liver necrosis may involve oxidation, it is suggested that alternate pathways of oxidative metabolism may be involved, one utilizing selenium, and the other utilizing tocopherol. If this were true, an organism would have to be deficient both in selenium and tocopherol before complete destruction of oxidative metabolism would occur. Investigation of the liver necrosis preventive effect of 200 selenium compounds and Factor 3 preparations has shown sodium selenite to be one of the most effective of the inorganic selenium compounds. The effective dose of selenium as Na_2SeO_3 that affords 50% protection (ED_{50}) to rats is 2 to 3 mg Se/100 g of diet (2).

The hepatotrophic action of Se (as an independent element or as a constituent of Factor 3) has been investigated with respect to its protective effect on hepatic necroses of rats due to the unilateral administration of powdered milk (3–6) or other hepatotoxic substances (7). However, the hepatostimulatory effects of selenium have not yet been sufficiently studied.

The above considerations prompted us to study the influence of Se on the regeneration of the liver, following subtotal hepatectomy. This test has been checked by a number of authors, as well as in the authors' laboratory, in the course of numerous experimental studies.

MATERIALS AND METHODS

Our experiments were made on 125 healthy albino rats of both sexes, having an average of 130 g body weight. These were divided into 4 groups and maintained under similar conditions. The rats were kept in cages made of wire netting, placed on a 1.5-m high metal stand. The litter placed under the animals was made of wood shavings. All 4 groups received

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[2] Schwarz, K., and W. Mertz 1960 Physiological effects of trace amounts of selenium. Conference on Physiological Aspects of Water Quality, Sept. 8–9, Washington, D. C., p. 79.

a semipurified (basal) diet, consisting of the following: casein (18), starch (63), yeast (8), sunflower oil (5), a mixture of salts (4) and agar-agar (2%) dissolved in twice-distilled water (dilution 1:1). Each animal received a daily dose of 25 to 30 g.

The control group consisted of 50 rats, maintained with a semipurified diet. Group Se (25 rats) received the same amount of semipurified diet, to which Na_2SeO_3 was added so that each animal received 3 μg Na_2SeO_3/100 g body weight (the Na_2SeO_3 was dissolved in twice-distilled water, w/w). Group E (25 rats) received the same amount of semipurified diet to which 50 mg vitamin E had been added/kg diet. Group Se+E (25 rats) also received the same amount of semipurified diet to which 1 μg Na_2SeO_3/100 g body weight and 50 mg vitamin E/kg diet had been added. Fresh food was prepared daily, prior to feeding the animals.

All rats were submitted to a subtotal hepatectomy, performed under ether anesthesia and strictly aseptic conditions. We extirpated the left lateral and median lobes of the liver, i.e., approximately 63.5% of the entire liver (8, 9). During the first day following the operation the animals were kept at a room temperature of about 27°. The administration of the semipurified diet was started in all groups 3 days before hepatectomy, being continued up to 3, 7, 11, 14 and 22 days after hepatectomy, when the animals were decapitated (10 from the control group and 5 each from the other groups). The experimental diets were similar in all groups of animals, but in the following groups these diets were supplemented as follows: group Se, 3 μg Na_2SeO_3/100 g body weight; group E, 50 mg vitamin E [3] /kg diet; group Se + E, 1 μg Na_2SeO_3/100 g body weight plus vitamin E, 50 mg/kg diet. The weight increase of the residual liver was calculated on the basis of Canzanelli's formula (10), taking into consideration the modifications of Dagradi et al. (11).

$$\% \text{ regeneration} = \frac{\text{wt regenerated liver} - \text{presumed wt residual liver after hepatectomy}}{\text{wt of 2 extirpated lobes}} \times 100.$$

According to the formula above, the weight of the regenerated liver was measured at the time the animals were killed, the weight of the 2 extirpated lobes being determined immediately after hepatectomy, using a precision balance. The presumed weight of the residual liver is calculated with the aid of tables, the data of which are based on the proportion of the animal's total body weight and liver weight.

Specimens of liver taken for microscopic analysis were always removed from equal distances from the site of resection of the liver. After fixation in 10% formalin the material was sectioned in part with a freezing microtome and the sections were stained with Sudan III for study of lipids; the rest was embedded in paraffin, sectioned at 7 μ and stained with hematoxylin and eosin and Masson's trichrome stain, for the study of mitoses.

The number of mitotic figures and of binucleated hepatocytes was expressed in percentage with up to 2,500 hepatic cells per animal being examined.

RESULTS

Macroscopical aspects of the hepatic regenerate. In the control group a brown-yellow color was noted, characteristic of a resected liver in the first phase of regeneration. After the seventh day this returned progressively to the dark-brown color of the normal liver. The livers of the animals of group Se were dark red in color and the lobular outlines were less visible. These phenomena persisted throughout the entire period of observation. In the livers of animals of groups E and Se+E the color of the hepatic regenerate was brown, with an almost unperceptible touch of yellow (more marked in group E). The contours of the lobules were more apparent than in the previous groups. In the rats treated with Se and vitamin E the weight increase of the residual liver was significantly greater on 7, 11 and 14 days after hepatectomy as compared with that of group C, main-

[3] Tableta, Pharmaceutical Works of Rumania (tablets of 0.03 g mixed with the diet).

tained exclusively with the semipurified basal diet (table 1). In the groups supplemented only with Se or vitamin E, significant increases were noted only on the eleventh day following hepatectomy.

Histological aspects of the liver. Control group. Three days after hepatectomy we noted a moderate sinusoidal hyperemia, numerous clear parenchymal cells, uniform cellular nuclei showing slight differences in staining, some sporadic histiocytic infiltrations, numerous large sudanophil droplets within the cytoplasm of cells pertaining to the central and intermediary areas, and small droplets in the areas corresponding to the periphery of hepatic lobules (fig. 1). Starting with the seventh day, a moderate hyperemia was noted throughout the whole period of observation, with the progressive decrease of fatty infiltration and its final disappearance on the fourteenth day. In other respects, the livers were completely normal.

Group Se. On the third day after hepatectomy, marked hyperemia was noted throughout the whole length of the hepatic parenchyma, with the appearance of numerous interstitial microhemorrhages

TABLE 1

Weight increase of regenerated liver of rats fed supplements of selenium, vitamin E, or selenium plus vitamin E (expressed as % of regenerated liver wt increase) (10, 11)

No. of days after hepatectomy	Controls (10) [1]	Selenium (5)	*P* value	Vitamin E (5)	*P* value	Selenium + Vitamin E (5)	*P* value
3	70 ± 3.70 [2]	79 ± 4.34	0.05	80 ± 7.04	0.30	76 ± 8.11	0.50
7	80 ± 3.18	89 ± 5.55	0.50	90 ± 12.84	0.60	127 ± 9.98	0.02
11	70 ± 4.65	93 ± 3.84	0.01	113 ± 12.82	0.02	133 ± 8.01	0.001
14	89 ± 4.84	111 ± 9.07	0.10	102 ± 9.42	0.30	124 ± 5.01	0.01
21	101 ± 2.25	105 ± 4.35	0.50	103 ± 6.46	0.70	109 ± 6.68	0.40

[1] Numbers in parentheses indicate number of animals/group.
[2] Mean ± SD.

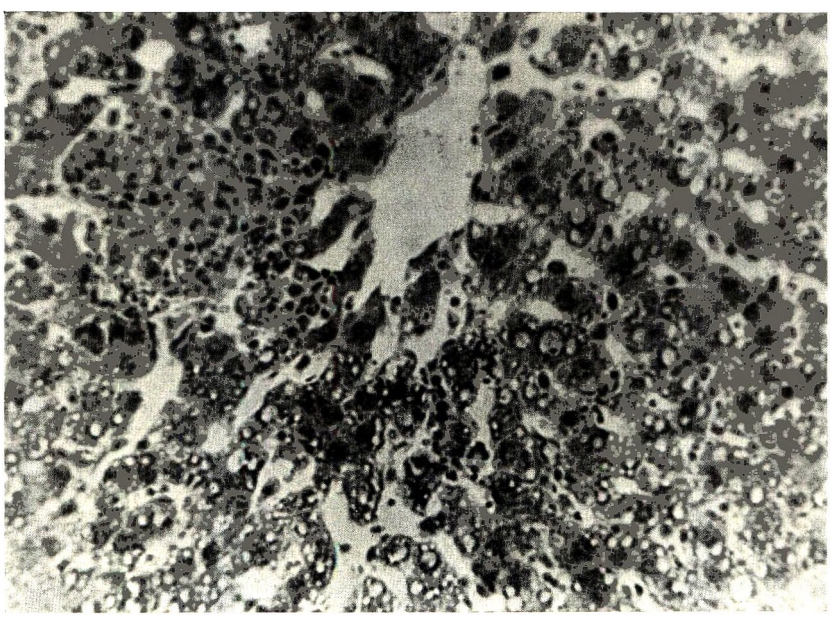

Fig. 1 Liver of control animal, 3 days after subtotal hepatectomy. Dilated sinusoids, moderate hyperemia, numerous large and medium-sized sudanophil droplets in the cytoplasm of hepatic cells; hystiocytic infiltration in the vicinity of the central vein. Sudan III stain. × 400.

as a result of which the liver acquired a motley appearance. Large sudanophil droplets were distributed evenly in the 3 areas of hepatic lobules (fig. 2). From the seventh day onward and throughout the period of observation, a sinusoidal hyperemia was noted, as well as parenchymatous microhemorrhages (in some cases sporadic and circumscribed, but more often dispersed throughout the preparations). The fatty infiltration disappeared on the eleventh day (fig. 3).

Group E. On the third day, the blood content of the liver was somewhat higher than in the controls. The hepatocytes were slightly hypertrophied, the nuclei of hepatic cells showing small differences in size and staining. Mitoses were observed more frequently than in the controls. The fatty infiltration was more pronounced and was limited only to the parenchymatous areas around the portal spaces (fig. 4). On the seventh day, the hyperemia became more pronounced, the number of binucleated cells increased, with some sporadic histiocytic infiltrations, and fatty infiltration was less evident than on the third day. On the eleventh day, the fatty substances disappeared completely from the liver.

Group Se + E. On the third day moderate hyperemia which affected the liver sinusoids was noted. Also noted were greatly hypertrophied hepatocytes, marked differences in the staining of the cytoplasm with frequent appearance of mitotic forms and binucleated cells, some sporadic histiocytic infiltrations and a number of large sudanophil droplets in the cytoplasm of the hepatocytes (without a definite topography) (figs. 5, 6). From the seventh day on and throughout the whole period of observation, we noted marked sinusoidal hyperemia (diffuse or territorial), binucleated cells in considerable proportions and some histiocytic infiltrations in the area of periportal spaces. Hepatic fatty infiltration disappeared on the seventh day.

The mean values of the mitotic index, as well as those of the binucleated hepatocytes are shown in table 2. This table also includes the degree of fatty infiltration, by which is meant the size and

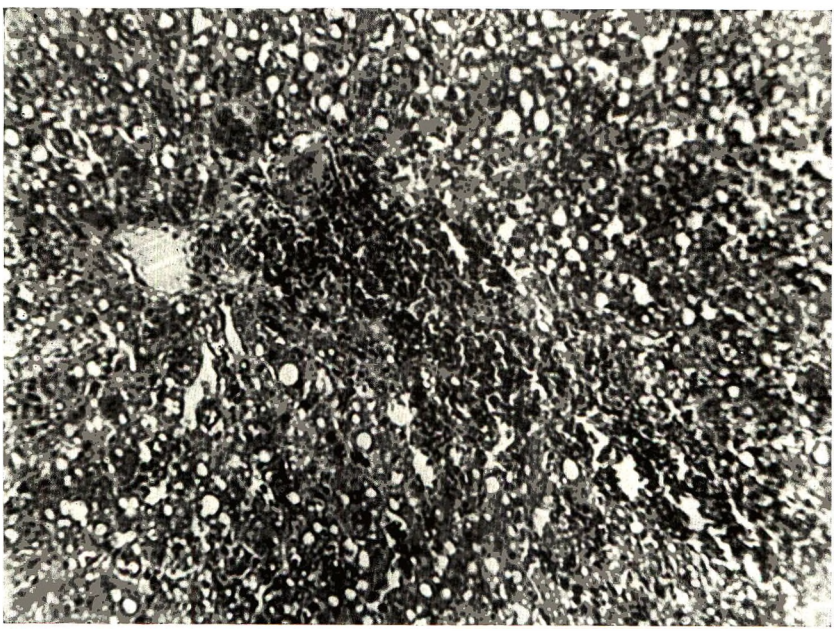

Fig. 2 The liver of animal treated with a daily dose of 3 μg/100 g body weight Na_2SeO_3, 3 days following subtotal hepatectomy. Circumscribed microscopical hemorrhages. Numerous large vacuoles (fatty) may be seen on the preparation. Masson's trichrome stain. $\times$ 400.

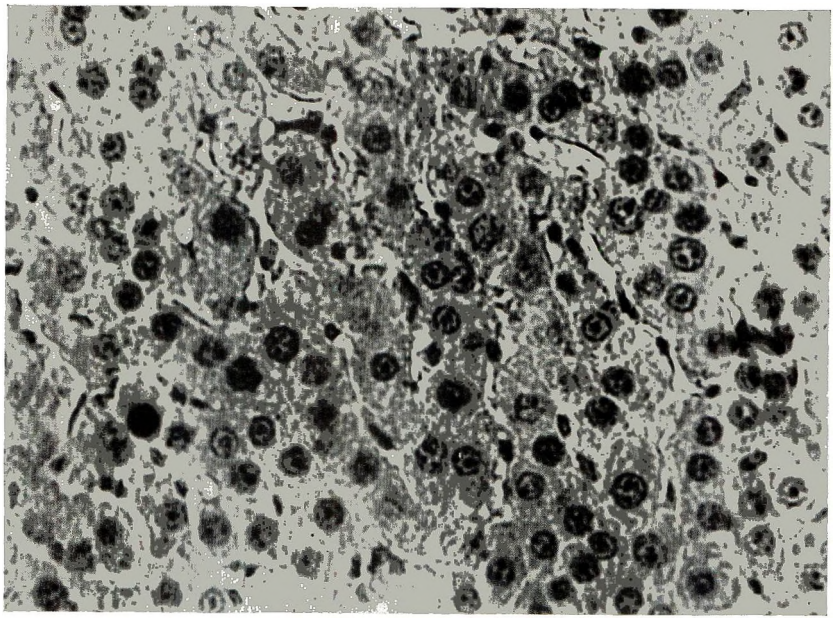

Fig. 3 The liver of animal treated with a daily dose of 3 μg/100 g body weight Na_2SeO_3, 14 days following subtotal hepatectomy. Marked sinusoidal hyperemia. Fatty vacuoles may not be seen. Hematoxylin-Eosin stain. $\times$ 500.

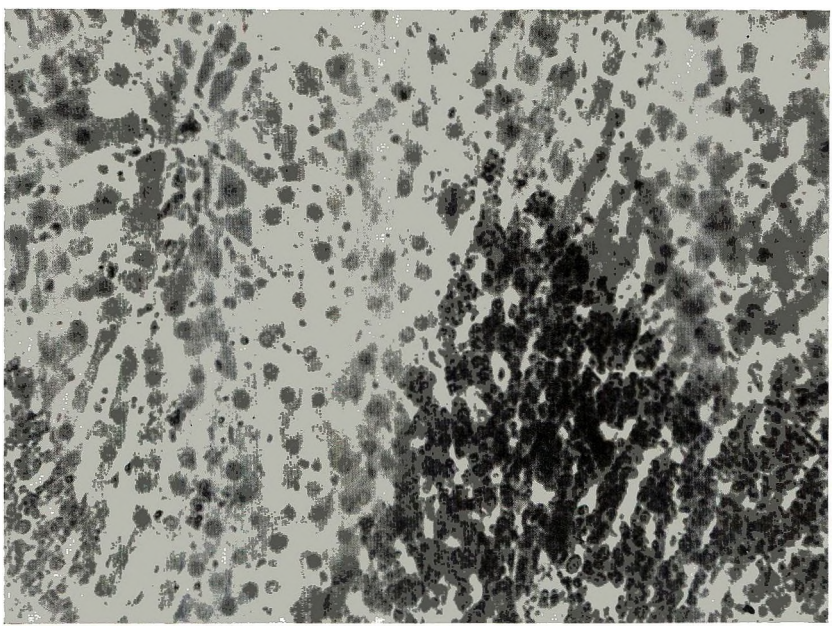

Fig. 4 The liver of an animal treated with daily dose of 50 mg vitamin E/1000 g food, 3 days after subtotal hepatectomy. Slightly swollen hepatic cells, uniform cell nuclei. The steatosis is limited exclusively to the periportal spaces. Sudan III stain. $\times$ 400.

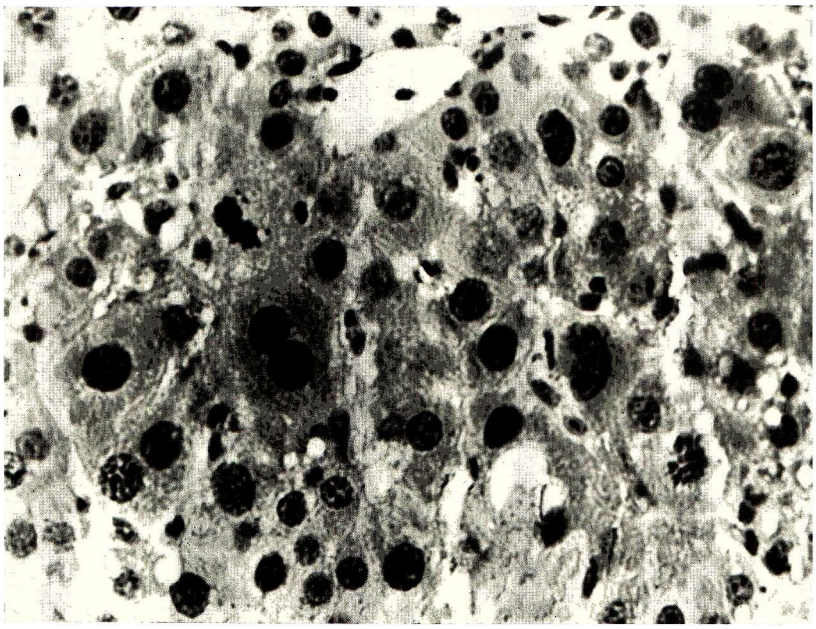

Fig. 5 The liver of animal treated with daily dose of 1 μg/100 g body weight Na_2SeO_3 combined with 50 mg vitamin E/1000 g food, 3 days after subtotal hepatectomy. Hypertrophied hepatocytes, the cell nuclei showing differences in size and staining. Frequent mitoses and numerous binucleated cells. Hematoxylin-Eosin stain. $\times$ 500.

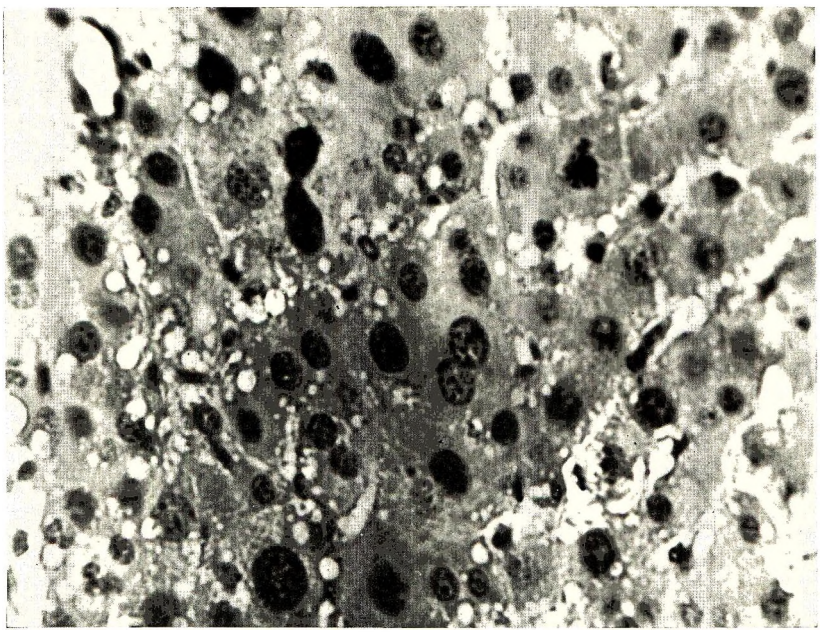

Fig. 6 The liver of an animal treated with a daily dose of 1 μg/100 g body weight Na_2SeO_3 combined with 50 mg vitamin E/1000 g food, 3 days following subtotal hepatectomy. Numerous hepatic cells showing large fatty vacuoles. Differences in the size and degree of staining of cell nuclei. Greater incidence of mitoses and of binucleated cells. H & E. $\times$ 500.

TABLE 2

Mitotic index, binucleated hepatocytes and degree of fatty infiltration of livers of rats fed supplements of selenium, vitamin E, or selenium plus vitamin E [1]

No. of days after hepatectomy	Controls			Selenium			Vitamin E			Selenium + vitamin E		
	Mitotic index [2]	Binucleated hepatocytes	Degree [3] of fatty infiltration	Mitotic index	Binucleated hepatocytes	Degree of fatty infiltration	Mitotic index	Binucleated hepatocytes	Degree of fatty infiltration	Mitotic index	Binucleated hepatocytes	Degree of fatty infiltration
3	4.41	9.90	++++	5.00	9.50	++++	7.88	10.07	++++	10.80	15.95	++++
7	1.60	9.50	+++	2.45	7.55	+++	9.20	15.60	+++	11.85	22.25	—
11	0.85	5.30	+	2.65	5.20	—	5.20	10.20	—	2.50	11.10	—
14	0.40	5.55	—	1.05	5.35	—	0.40	8.60	—	0.30	10.45	—
21	—	5.40	—	—	4.05	—	—	5.10	—	—	5.60	—

[1] Mean values.
[2] Percentage of the hepatic cells in the various stages of the mitotic process, reported to 2,500 hepatic cells/animal.
[3] Size and distribution of the neutral fatty droplets stained with Sudan III, observed microscopically. Crosses indicate degree of fatty infiltration judged on the basis of 50 sections/animal.

distribution of the neutral fatty droplets stained with Sudan III and observed microscopically. The number of crosses indicates the degree of fatty infiltration, as judged on the basis of 50 sections per each animal.

DISCUSSION

The microscopic analysis of the specimens indicated that the separate administration of Se in daily doses of 3 μg/100 g body weight in amounts somewhat higher than the protective dose, in addition to the activation of the mitotic processes, produced an intensification of the hepatic circulation and capillary lesions. The dark red color and the less evident outlines of the lobules, characteristic of the hepatic regenerate of rats in this series, may be explained by the considerable number of parenchymal microhemorrhages, thus providing evidence which indicates increased permeability of the hemato-tissular barrier in the liver. The administration of smaller doses of Se, 1 μg/100 g body weight combined with vitamin E, produced a marked weight increase of the hepatic regenerate, as well as an impressive intensification of both the mitotic divisions and an increase of binucleated cells (particularly in the course of the first 11 days). This supplement also had a stimulatory effect on hepatic circulation, but produced no vascular lesions. The lack of interstitial microhemorrhages in group Se+E is a confirmation of this fact. Vitamin E, given separately, evidently stimulates the increase of mitoses and binucleated cells, but not to the same degree as in those cases when its administration is combined with selenium. This fact agrees with the observations of other authors who have noted that vitamin E has a positive effect on the regeneration of the liver (12). It is also known that in rats fed a protein-deficient and vitamin E-free diet, the development of liver necroses occurs, which may be counteracted by the administration of vitamin E (13).[4] Such selective effects were also

[4] György, P. 1946, 1949, 1952 In: Liver Injury, Transactions of the Fifth, Eighth and Eleventh Conferences. Josiah Macy, Jr., Foundation (cited in Die Leber, H. Popper and F. Schaffner, eds. Georg Thieme Verlag, Stuttgart, Germany, 1961, p. 787, ref. no. 1.319).

noted in our investigations on the action of other hepatostimulatory factors (14-17).

The hepatostimulatory effects of Se are also shown by the faster rate at which the hepatic fatty infiltration disappears, as compared with the rate of disappearance in the controls. In our previous communications (18–20) we demonstrated that the sudanophil droplets observed in the hepatic cells constitute one of the main sources of energy of the karyokinetic process occurring in the liver, exhausting itself in proportion to the degree of intensification of the latter. This fact is in accordance with observations in the present study.

CONCLUSIONS

Our observations may be summarized as follows:

1. Na_2SeO_3 given in combination with vitamin E produces a considerable intensification of the processes leading to the regeneration of rat liver, following subtotal hepatectomy. These effects are much more pronounced than in the cases where the above 2 substances are given singly to various groups of animals.

2. Na_2SeO_3 has a selective influence on the mitotic process, while on the binucleated cells it has only a slight influence. Combined with vitamin E, it clearly stimulates the different forms of cell division in the liver.

3. It appears that the physiological dose necessary for the stimulation of liver regeneration is about 1 μg/100 g body weight. Administered in larger quantities, the selenium not only stimulates the karyokinetic process, but also produces certain toxic effects on the vascular system in the form of parenchymatous microhemorrhages.

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Effect of Different Essential Amino Acid Deficiencies on Amino Acid Pools in Rats [1,2]

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ABSTRACT The effects of single essential amino acid deficiencies on the quantities of free amino acids present in plasma, liver, and muscle were investigated. Rats were force-fed for 3-day periods diets devoid in either leucine, isoleucine, valine, or threonine. At 8 hours postprandial, the amino acid limiting in the diet, without exception, was decreased in concentration in plasma and liver tissue. However, leucine and threonine showed greater decreases in concentration in the amino acid pools when these amino acids were omitted from the diet than the decreases for isoleucine or valine when these amino acids were deficient. The changes in amino acid concentrations in liver tissue were correlated with those in plasma to a greater extent than the alterations in amino acid values of muscle tissue. When rats were fed the diet without leucine, significant elevations occurred in many of the amino acids in the amino acid pools and, particularly in isoleucine and valine.

There is considerable experimental evidence to show (1–4) that an increase or a decrease in the dietary level of an essential amino acid results in a corresponding change in the concentration of that amino acid in plasma. In addition, the values for other amino acids in plasma might also be altered. However, it appears possible that a severe dietary deficiency of one particular amino acid would have a greater effect on plasma amino acid levels than a deficiency due to another amino acid and that changes in the free amino acid concentrations of liver and muscle might not be correlated with those of the plasma. The effects of single essential amino acid deficiencies can be investigated only in experiments where diets containing purified amino acids are used and nutrient intake is controlled. Very few studies of this nature have been conducted. Sidransky and Verney (5) reported that only minor changes occurred in the amino acid levels in liver tissue of rats force-fed amino acid mixtures devoid of a single essential amino acid. Roberts (6), using the paired-feeding technique, reported that rats fed a diet deficient in phenylalanine exhibited alterations in the plasma levels of several essential and nonessential amino acids. Dean and Scott (7) fed chicks diets deficient in either lysine or valine for 6-hour periods and observed a marked lowering of the limiting dietary amino acid and an increase in most of the other amino acids in plasma.

The present studies were conducted in rats to compare the effects of several different essential amino acid deficiencies on the amino acid levels in plasma during the postabsorption period and to determine whether the alterations in plasma amino acids were associated with concomitant changes in liver and muscle tissue. In these studies, rats were force-fed diets lacking in either leucine, isoleucine, valine, or threonine.

EXPERIMENTAL

Male rats (210 g) of the Sprague-Dawley strain were allotted at random to the treatments in each experiment. The control animals received the complete amino acid (CAA) diet. The experimental animals received diets similar to the CAA diet but devoid of a single essential amino acid, either leucine (−Leu), isoleucine (−Ileu), valine (−Val), or threonine (−Thr). Glucose was substituted for the missing essential amino acid.

The composition of the CAA diet was as follows: (%) L-amino acids in a mixture

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simulating casein, 21.46; glucose, 60.34; corn oil,[3] 10.00; mineral mix,[4] 4.00; cellulose,[5] 2.00; and vitamin mixture,[6] 2.20. The composition of the amino acid mixture was as follows (%): tryptophan, 0.25; lysine·HCl, 1.81; arginine·HCl, 0.89; histidine·HCl, 0.68; phenylalanine, 0.98; leucine, 1.82; isoleucine, 1.19; valine, 1.34; methionine, 0.56; threonine, 0.78; serine, 1.22; glutamic acid, 4.18; tyrosine, 1.05; cystine, 0.27; proline, 2.13; aspartic acid, 1.34; alanine, 0.61; and glycine, 0.36. The vitamin mixture contributed the following amounts (mg/100 g diet): vitamin A, 0.59 (1980 IU); vitamin D, 0.006 (220 IU); α-tocopherol, 11.0; ascorbic acid, 99.0; inositol, 11.0; choline chloride, 165.0; menadione, 5.0; *p*-aminobenzoic acid, 11.0; niacin, 9.9; thiamine·HCl, 2.2; riboflavin, 2.2; Ca pantothenate, 6.6; biotin, 0.04; folic acid, 0.2; pyridoxine·HCl, 2.2; and vitamin B_{12}, 0.003.

In experiment 1, to prevent alterations occurring in the amino acid pools due to a decreased food intake, the rats were fed by stomach tube using the feeding procedure of Sidransky and Verney (5). The rats received 18.6 g of diet per day (6.2 g/feeding). For the tube feeding, the diets were mixed with tap water, homogenized in a Waring Blendor, and diluted to a total volume in milliliters that was equivalent to the grams of dry diet. To accustom the animals to the feeding procedure, all animals were prefed an 18% casein diet, prepared as described above, for 2 days prior to administering the experimental diets containing the amino acids. These diets were then fed for 3 days. The rats were anesthetized with ether at 13 hours after the last intubation and blood was removed for subsequent amino acid analysis.

In experiment 2, two modifications of the feeding procedure were made. The rats were fed 4 times daily, at 8:00 A.M, 1:00 PM, 5:30 PM, and 10:30 PM and they received 20 g of diet per day (5 g/feeding). They were killed at 8 hours postprandial and blood and liver and muscle were removed for amino acid analysis. In a preliminary experiment, an attempt was made to define a time interval after feeding during which the plasma amino acids remained in steady state equilibrium with the tissues after feeding. Rats were fed 5 g of the CAA diet and killed at 2-hour intervals over a 10-hour period. It was found that most of the amino acids reached their highest levels in plasma 2 or 4 hours after feeding and were greatly decreased by 6 hours, with a further small decline by 8 hours. At 10 hours, the values were very similar to those of the 8-hour period. Therefore, the 8-hour interval was selected for the killing time as representing a post-absorption steady state concentration of amino acids under these experimental conditions.

Amino acid analyses were performed by ion-exchange chromatography (8) on deproteinized supernatants of plasma, liver and muscle (gastrocnemius). These supernatants were prepared with some modifications of the procedure of Stein and Moore (9) for plasma, and the method of Tallan et al. (10) for the other tissues. A 3% sulfosalicyclic acid solution (11) was substituted for picric acid in preparing the samples. After the blood was removed by cardiac puncture, the liver and muscle were quickly excised, freed of fat and connective tissue, weighed, and cut into small pieces. For the liver, 1 g was homogenized immediately (Potter-Elvehjem homogenizer) with a tenfold amount of acid. For the muscle, 0.5 g was immediately ground in the Waring Blendor with a 20-fold amount of acid. All samples were centrifuged promptly for 10 minutes at 2000 rev/min and stored at − 20° until analysis was performed. Asparagine and glutamine were not separated chromatographically and are reported as a mixture. The data were statistically analyzed by methods described by Snedecor (12).

RESULTS

Growth rate and amino acids in plasma (exp. 1, table 1). All groups of animals fed the amino acid-deficient diets, except those receiving the −Leu diet, lost weight during the 3-day experimental period. The group receiving the CAA diet gained weight but at a slow rate for this type of

[3] Mazola, Corn Products Company, Argo, Illinois.
[4] Hegsted Mineral Mix, Nutritional Biochemicals Corporation, Cleveland, (Hegsted et al., J. Biol. Chem., *138*: 459, 1941).
[5] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[6] The vitamin fortification mixture was obtained from Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Plasma amino acid levels [1] and growth rate of rats fed diets devoid of a single essential amino acid (exp. 1)

Amino acid	Diet					Error variance [3]	F ratio [4]
	Complete amino acid (6) [2]	−Leu (5)	−Ileu (5)	−Val (5)	−Thr (3)		
Threonine	26.0	16.0	20.4	24.8	8.4 [5]	21.12	9.62**
Valine	6.5	26.7 [5]	7.2	8.4	8.1	83.62	5.56**
Isoleucine	4.7	9.5	4.3	4.1	4.4	12.06	2.17
Leucine	9.3	4.4 [5]	6.6	6.7	7.5	1.89	8.31**
Methionine	2.4	1.7	2.8	3.3	3.1	0.48	4.17*
Phenylalanine	2.9	3.0	3.4	3.8	3.3	0.45	1.54
Total mean body wt gain, g	5.2	1.2	−3.6 [5]	−2.2 [5]	−5.0 [5]	8.26	9.73**

[1] Values are the means in μmoles/100 ml from individual determinations.
[2] Numbers in parentheses indicate number of rats/treatment.
[3] df for error variance = 19.
[4] * $P < 0.05$; ** $P < 0.01$.
[5] Indicates significant difference from complete amino acid diet values.

diet. Some rats on each treatment had occasional diarrhea, a condition that was not observed in the next experiment when a different feeding procedure was introduced.

For the groups of animals fed the diets from which an amino acid had been omitted, except those fed the −Val diet, the mean postabsorption plasma amino acid value of the amino acid missing from the diet was lower than the value in rats receiving the CAA diet. However, only threonine and leucine were significantly decreased under the conditions of this experiment.

In addition to these changes, the animals receiving the −Leu diet showed a significant increase in valine as compared with the values of all other groups. The level of isoleucine was elevated and the methionine was depressed, but the changes were not significant.

The nonessential amino acids were not tabulated for this experiment because only minor differences were observed among the groups fed the different diets.

Growth rate and amino acids in plasma (exp. 2, table 2). In this experiment, the animals fed the CAA diet gained twice as much weight as the animals receiving the same diet in experiment 1. This result was probably due to the improved feeding procedure. Only the animals fed the −Thr diet were unable to maintain their initial body weight during the 3-day period. All other groups gained less weight than the controls, with the rats fed the −Leu and −Val diets showing the least depression in growth rate.

Again in this experiment, administering diets from which an amino acid had been omitted resulted in a lower mean level of that amino acid in the plasma as determined 8 hours postprandial. The levels of threonine and of leucine in plasma were significantly lower in rats fed the −Thr and −Leu diets, respectively, than in rats fed the CAA diet.

When the −Leu diet was fed, significantly higher values for many of the amino acids in plasma were obtained. The levels of valine, isoleucine, phenylalanine, methionine, alanine, asparagine-glutamine and proline were all significantly increased in comparison with values from rats fed the CAA diet. In particular, isoleucine and valine values showed extreme elevations.

When the amino acid-deficient diets other than the −Leu diet were fed, generalized increases in plasma amino acid levels were not observed but occasional changes were noted. The rats fed the −Ileu diet showed a decreased theronine level and cystine was increased as compared with values for rats fed the CAA diet. The rats given the −Val diet showed increased levels of phenylalanine, tyrosine, cystine and asparagine-glutamine. When the −Thr diet was administered, a significant increase in the level of phenylalanine resulted.

TABLE 2

Amino acid levels [1] in the plasma and growth rates of rats fed the various diets (exp. 2)

Amino acid	Diet					Error variance [2]	F ratio [3]
	Complete amino acid	— Leu	— Ileu	— Val	— Thr		
Threonine	29.4	35.8	19.2	32.4	7.7 [4]	41.54	15.76**
Valine	10.9	72.6 [4]	12.1	5.7	11.6	15.37	256.85**
Isoleucine	5.3	27.1 [4]	4.2	6.6	5.7	5.75	81.92**
Leucine	10.2	4.8 [4]	9.3	10.3	9.2	4.32	5.98**
Methionine	2.2	3.9 [4]	2.8	3.2	3.2	0.64	3.05*
Phenylalanine	3.2	7.2 [4]	4.7	5.2	5.8 [4]	1.32	7.91**
Tyrosine	9.4	17.6	13.9	18.5	8.1	27.20	4.02*
Cystine	4.9	9.2	12.1 [4]	7.9	6.5	8.10	4.65**
Serine	32.5	30.3	27.4	28.6	28.4	29.48	0.68
Asparagine-glutamine	30.1	58.1 [4]	26.4	42.2	33.4	192.25	4.16*
Proline	13.1	21.5 [4]	14.0	17.7	14.6	8.81	6.71**
Glutamic acid	10.7	21.2	15.9	11.5	10.7	99.35	1.94
Glycine	17.6	24.2	15.4	16.8	24.6	38.93	2.44
Alanine	44.8	78.0 [4]	47.0	45.3	57.1	262.52	3.78*
Lysine [5]	56.4	57.9	44.1	61.2	69.2		
Histidine [5]	4.3	5.9	4.5	6.8	6.3		
Arginine [5]	12.7	10.0	10.6	12.3	16.3		
Total mean body wt gain, g	12.0	8.0	4.0 [4]	7.0	−0.3 [4]	11.62	13.39**

[1] Values are the means in μmoles/100 ml from individual determinations of 5 rats/treatment except for the basic amino acids.
[2] df for error variance = 20.
[3] * $P < 0.05$; ** $P < 0.01$.
[4] Indicates significant difference from complete amino acid diet values.
[5] Values are the means from two determinations of pooled samples from 5 rats. Data not analyzed statistically.

Amino acids in liver and muscle (exp. 2, table 3). The amino acid content of liver tissue from rats fed the amino acid-deficient diets showed the same changes in comparison with the control animals as observed for plasma. Values for threonine and leucine in liver were lowered significantly in animals fed the −Thr and −Leu diets, respectively. The mean values for valine and isoleucine were also decreased, but not significantly in rats fed the −Val and −Ileu diets, respectively. In rats fed the −Leu diet, the levels of the other branched-chain amino acids and alanine were increased significantly.

As compared with the control group, the animals fed the amino acid-deficient diets showed fewer differences in amino acid composition in muscle tissue than those evidenced in plasma and liver tissue. Threonine and leucine were significantly lowered in concentration in the muscle of animals receiving the −Thr and −Ileu diets, respectively. However, the mean value of isoleucine in muscle was slightly increased when the −Ileu diet was fed and likewise the valine value was higher in rats receiving the −Val diet. The administration of the −Leu diet resulted in a significant increase in the levels of valine and isoleucine and in addition, the values of many other amino acids in muscle were also elevated.

DISCUSSION

These results obtained with diets containing amino acid mixtures are in agreement with data from low protein diets in that the plasma and tissue concentrations of the limiting dietary amino acid were decreased in all instances (13, 14). However, it was found that the feeding of low protein diets in both animals and humans is associated with increases in the levels of some of the nonessential amino acids in plasma and a resultant lower ratio of essential to nonessential amino acids (15, 16). These changes were not observed with any of the amino acid-deficient diets studied here. A possible explanation for this difference in response might be related to the calorie intake. It has been found that under

some conditions the caloric requirements are increased for diets containing amino acids (17). It is possible that in animals receiving amino acid mixtures there is a limited availability of the carbon chain for the synthesis of nonessential amino acids and hence these amino acids are not increased in plasma.

There is some evidence in the experiments reported here that the effects of dietary amino acid deficiencies on plasma and tissue amino acid levels are dependent to some degree upon the time interval between feeding and measuring the amino acid concentrations. The data presented in tables 1 and 2 indicate that animals receiving the CAA diets had similar plasma amino acid concentrations at 13 hours and 8 hours after feeding. However, when the differences in amino acid values in the plasma of animals fed the amino acid-deficient diets are compared with the values of the animals given the CAA diet, the differences are much greater at 8 hours (table 2) than at 13 hours (table 1) after feeding. In the experiments reported by Sidransky and Verney (5) animals fed various diets devoid of an essential amino acid, in comparison with control animals, were observed to have only minor differences in amino acid concentrations in liver tissue obtained 18 hours after feeding. It appears that the alterations in amino acid levels resulting from dietary amino acid deficiencies are lessened as the postprandial time is increased. This situation may not pertain, however, if the labile protein reserves of the animal have been

TABLE 3

Amino acid levels [1] in liver and muscle of rats fed the various diets

Amino acid	Diet					Error variance [2]	*F* ratio [3]
	Complete amino acid	— Leu	— Ileu	— Val	— Thr		
Liver							
Threonine	95.1	47.2	60.2	112.5	39.8 [4]	735.33	6.73**
Valine	23.7	73.1 [4]	14.9	17.2	18.7	93.38	33.53**
Isoleucine	14.2	26.8 [4]	7.9	11.7	11.4	16.92	20.68**
Leucine	30.6	14.9 [4]	21.3	26.6	23.4	37.77	4.58**
Methionine	6.9	7.6	6.6	7.6	7.0	10.86	0.109
Phenylalanine	9.6	8.8	8.2	9.5	9.8	3.58	0.60
Tyrosine	17.9	20.9	14.0	23.3	12.8	37.37	2.67
Aspartic acid	171.3	120.6	147.4	110.1 [5]	233.6	3920.97	3.09*
Serine	127.2	109.0	65.2 [4]	155.4	150.2	931.22	7.16**
Asparagine-glutamine	300.2	483.7	229.5	427.8	328.1	35617.18	1.46
Glutamic acid	332.9	334.5	303.1	277.0	268.0	4584.84	1.03
Glycine	230.9	287.4	198.5	239.1	232.2	1951.37	2.61
Alanine	431.2	659.2 [4]	420.6	429.3	500.4	12124.25	4.20*
Muscle							
Threonine	90.5	70.9	77.2	113.2	29.4 [4]	257.64	18.40**
Valine	13.0	71.3 [4]	22.7	15.4	19.3	290.08	9.46**
Isoleucine	8.5	29.5 [4]	9.5	11.7	11.5	5.90	63.87**
Leucine	13.7	10.0	20.4	19.4	16.8	41.06	2.22
Methionine	4.1	2.1	2.7	3.0	5.1	8.68	0.71
Phenylalanine	5.8	8.3	9.3	13.0	10.0	33.52	1.04
Tyrosine	15.2	24.4	29.2	45.9	22.2	574.50	1.15
Aspartic acid	25.2	33.4	32.0	25.4	24.3	33.13	2.76
Serine	160.3	98.5 [4]	132.9	144.0	120.0	634.17	4.36*
Asparagine-glutamine	320.0	306.7	323.1	418.3	309.3	6440.09	1.70
Proline	34.6	58.2	56.5	67.1	41.1	313.71	2.79
Glutamic acid	83.4	107.8	95.3	108.1 [5]	80.7	291.48	2.91*
Glycine	219.7	257.7	245.3	265.2	274.6	1681.72	1.34
Alanine	295.9	350.3	394.8	355.7	367.4	7162.28	0.91

[1] Values are the means in μmoles/100 g wet weight from individual determinations of 5 rats/treatment.
[2] df for error variance = 20.
[3] * $P < 0.05$; ** $P < 0.01$.
[4] Indicates significant difference from complete amino acid diet values.
[5] Indicates significant difference from — Thr values.

depleted. The changes in amino acid patterns resulting from dietary regimens should be studied further in relation to postprandial time and the severity of the amino acid deficiency state.

This study has provided evidence that the free amino acid pools in muscle respond somewhat differently than those in liver to diets deficient in an essential amino acid. Changes in amino acids in liver tissue appeared to have a higher correlation with the alterations observed in amino acid concentrations in plasma than did muscle tissue. For example, in liver the isoleucine, valine, leucine, and threonine levels were reduced in value for animals fed diets deficient in these amino acids as compared with values for the control animals. However in muscle, isoleucine and valine were increased slightly in concentration but leucine and threonine were decreased when these amino acids were omitted from the diet.

The results obtained with this study indicate that specific amino acid deficiencies invoked under constant experimental conditions, including food intake, produce varying responses in the amino acid pools. In part, these varying responses relate to the degree by which the amino acid deficient in the diet is reduced in concentration in plasma and tissues. Throughout this study, administering the —Thr and —Leu diets produced greater decreases in values for the limiting dietary amino acid in plasma and tissues than did either the —Val or the —Ileu diets. The threonine level was decreased to the greatest extent in the amino acid pools and the threonine-deficient animals also evidenced the greatest depression in growth rate, but for the other deficient groups, decreases in amino acid concentrations in plasma and tissues and growth rate were not well correlated.

Administering diets from which an essential amino acid was omitted caused alterations of amino acid levels in plasma and tissues other than the amino acid missing in the diet. The —Leu diet was distinct from all other amino acid-deficient diets tested in this respect. It is not known whether the increased concentrations that occurred for many of the amino acids, (especially isoleucine and valine) resulted in the primary instance from alterations in metabolism or in transport. Rogers et al. (18) fed rats diets containing excess amounts of leucine and observed decreases in plasma concentrations of isoleucine and valine, the opposite effect of the results reported here with a —Leu diet. These observations suggest that leucine may have some special role in maintaining amino acid homeostasis and there is some evidence that this may occur through hormonal mediation (19).

Lyman and Wilcox (20) have shown that animals fed diets devoid of an essential amino acid for 3 days have a normal proportion of total body water. Assuming a normal distribution between intra- and extracellular space, calculations (21, 22) can be made for the intracellular concentrations of amino acids in the liver and muscle cells. The results expressed on the basis of intracellular water content show the same relations in amino acid levels among animals receiving the control and amino acid-deficient diets as those presented for whole tissue (table 3). How-

TABLE 4

Calculated distribution ratios[1] of the essential amino acids between intracellular muscle water and plasma of rats receiving the various diets

Amino acid	Diet				
	Complete amino acid	— Leu	— Ileu	— Val	— Thr
Threonine	4.3	2.7	5.7	4.8	5.2
Valine	1.6	1.2	2.5	3.7	2.2
Isoleucine	2.2	1.4	3.1	2.3	2.7
Leucine	1.8	2.8	3.0	2.5	2.5
Methionine	1.2	0.8	1.2	1.1	1.9
Phenylalanine	2.4	1.5	2.7	4.1	2.3

[1] Plasma was assumed to contain 92% H_2O; muscle was assumed to contain 76% H_2O and of this, 84% is intracellular.

ever, when the distribution ratios of the essential amino acids between intracellular muscle water and the plasma (table 3 and 2) are calculated, some differences occur, as summarized in table 4. For rats fed each of the amino acid-deficient diets, the muscle-to-plasma ratio of the amino acid omitted from the diet is higher than for rats fed the control diet. This indicates a compensatory concentration within the muscle cell of the amino acid that is deficient in the diet. With the other amino acids, the distribution ratios between muscle and plasma tended to be higher for rats fed the amino acid-deficient diets, except for those fed the −Leu diet where the ratios were lower. These data suggest that there is no difficulty in amino acid transport in the amino acid deficiencies investigated here with perhaps the exception of a leucine deficiency. It is possible that the low muscle-to-plasma distribution ratios which occur with a leucine deficiency are secondary to the extremely high concentrations of isoleucine and valine. The effect of leucine on amino acid transport should be investigated further.

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Digestion of Potato Starch by Larvae of the Flour Beetle, *Tribolium castaneum*

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ABSTRACT *Tribolium castaneum* larvae do not develop when fed a diet in which potato starch is the sole source of carbohydrate. The physical resistance of the intact starch granule hinders digestion but boiling and disrupting the starch granules only partly improves it. The esterified phosphate present in the β-amylase limit-dextrin (β-dextrin) of potato starch affects the pattern of amylatic degradation by *Tribolium* amylase, and previous dephosphorylation in vitro slightly improves subsequent utilization by the larvae in vivo and digestion by larval amylase in vitro. The endogenous larval midgut phosphatase is apparently not effective in carrying out this dephosphorylation in vivo. The comparative action patterns (the distribution of products of amylolysis as evidenced by chromatographic means) of *Tribolium* amylase and of salivary α-amylase on β-dextrins differ. The nutritional significance of the variant modes of action and products of hydrolysis is expressed in the observation that predigestion in vitro of potato starch β-dextrin by salivary α-amylase results in the complete utilization of the hydrolyzed β-dextrin by *Tribolium* larvae in vivo.

A previous investigation on the utilization of starch by larvae of the Rust Red flour beetle, *Tribolium castaneum* (Herbst), indicated the applicability of this biological system in assessing the nutritional value of starches and starch fractions (1). Potato starch, which is known to be of poor nutritional value for poultry (2) and rats (3), apparently affected the development of *Tribolium* in a similar manner. This was attributed in part to the resistance of the intact starch granule to digestion, as boiled potato starch did support suboptimal larval development. The observation that larvae of *Tribolium* could not utilize the β-amylase limit-dextrin (β-dextrin) core of potato starch led to the suggestion that this could be attributed to the presence of esterified phosphorus in that fraction. This suggestion was supported by the observation that the β-dextrin of amylopectin from other sources did afford optimal growth (1).[1] The present study was an attempt to ascertain whether the esterified phosphate is indeed implicated in the poor utilization of potato starch by *Tribolium* larvae and to determine the role of amylase specificity in the overall resistance.

MATERIALS AND METHODS

Larvae of *T. castaneum* (Herbst) were of the same strain used in previous experiments (1). They were reared with whole wheat flour in glass jars at the optimal temperature of 32° (8).

In vitro enzymic experiments. Larval enzyme solutions were prepared as described previously (9) at concentrations in accord with preliminary determined, linear phosphatase activity. The enzyme solutions were prepared in cold distilled water and filtered through cotton wool. Enzyme solutions were freshly prepared before each experiment.

Phosphatase activity was assayed on *p*-nitrophenyl phosphate (10, 11) in a 2-ml reaction mixture. Reaction mixtures were buffered with 0.025 M Tris-maleate buffer. A period of 5 minutes' equilibration preceded the 20-minute reaction at 37°. Reactions were terminated by addition of 1 ml of 0.2 N NaOH. Optical density was determined with a Bausch and Lomb Spectronic 20 spectrophotometer at 420 mμ. One unit of phosphatase activity is defined as activity liberating one micromole of *p*-nitrophenol per minute, under defined conditions, at 37°.

The hydrolysis of several starch fractions by *Tribolium* amylase was assayed in 0.0025 M acetate buffer, pH 5.2, plus

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[1] Potato starch is known to contain an appreciable amount of phosphorus esterified on the 6-position of the glucose residues (4,5) and concentrated in the β-dextrin of the amylopectin fraction (6,7).

0.0025 M $CaCl_2$. The action patterns (the distribution of products of amylolysis as evidenced by chromatographic means) were compared with those obtained with purified human salivary α-amylase [2] in 0.0025 M phosphate buffer pH 7.4 plus 0.0025 M NaCl. Ascending thin-layer chromatography (TLC) of the degradation products was performed in a solvent system of ethyl acetate: *iso*-propanol:*n*-butanol:water (80:40:20:20) (12), on 20- by 20-cm glass sheets plated with Keiselgur G,[3] and color was developed with acidified anisaldehyde in ethanol (13).

Growth experiments with Tribolium larvae. The composition of the diets used is shown in table 1. Amounts of 0.25 g diet were assayed in 5 replicates consisting of 10 newly hatched larvae per replicate. After 14 days at 32° the larvae were removed from their diets, and each group was weighed and returned to its container for further observations.

TABLE 1

Composition of basic experimental diets for Tribolium larvae

	%
Carbohydrate	80
Gluten [1]	7
Cellulose [2]	6
Brewer's yeast [1]	5
Salt mixture no. 2, USP [1]	1
Cholesterol [3]	1

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Alphacel, Nutritional Biochemicals Corporation.
[3] Reidel-De Haën, AG., Hanover, West Germany.

[2] Kindly supplied by Miss R. Shainkin of the Department of Biochemistry, Faculty of Agriculture, Rehovot.
[3] E. Merck AG., Darmstadt, West Germany.

TABLE 2

Effect of modified potato starch fractions on larval development of Tribolium

Fraction assayed	Avg wt of larvae	Larval survival (14 days)	Ultimate survival and pupation
	mg	%	%
Potato starch (raw) [1]	0	0	
Potato starch (boiled) [2]	0.44	72	56
Potato starch β-amylase-limit dextrin [3]	0	0	0
Phosphatase-treated β-amylase-limit dextrin of potato starch, I [4]	0.2	16	10
Phosphatase-treated β-amylase-limit dextrin of potato starch, II [5]	0.1	28	26
Phosphatase-supplemented β-amylase-limit dextrin of potato starch [6]	0	0	0
Potato starch extensively digested with salivary α-amylase [7]	1.87	96	96
Rice starch control [8]	1.84	98	98

[1] Allied Chemical and Dye Corporation, New York.
[2] Prepared by boiling potato starch in water and drying under reduced pressure in rotary evaporator.
[3] Prepared according to Applebaum and Konijn (1).
[4] Prepared from a boiled 2% solution of β-amylase-limit dextrin in 0.05 M tris-maleate buffer pH 8.0. Calf intestinal alkaline phosphatase (Mann Research Laboratories, Inc., New York) was added at a concentration of 50 μg/ml and incubated at 35° for 22 hours under toluene. The solution was dialyzed overnight against running tap water and subsequently against distilled water. The precipitate which formed was centrifuged and dried over P_2O_5.
[5] The dialyzed supernatant remaining from no. 4 was dried under reduced pressure in a rotary evaporator and overnight against P_2O_5.
[6] Calf intestine alkaline phosphatase (Mann Research Laboratories, Inc.) was added in the amount of 4 mg/replicate.
[7] Prepared from a boiled 4% solution of potato starch in 0.02 N phosphate buffer pH 7.0 + 0.01 N NaCl. Highly purified human salivary α-amylase (21) was added at a concentration of 10 μg/ml and incubated at 32° for 9 hours under toluene. The solution was then filtered through Whatman no. 1 filter paper and boiled for 2 minutes to inactivate the amylase. The solution was treated 3 times with activated charcoal powder, and the pooled charcoal adsorbates were eluted twice with with 50% ethanol. The eluates were pooled and dried under reduced pressure in a rotary evaporator.
[8] The British Drug Houses, Essex, England.

RESULTS AND DISCUSSION

Boiled potato starch slightly supported development (table 2). The method of drying the preparation is reflected in a poorer developmental response than that previously obtained from a lyophilized preparation (1).

Alkaline-phosphatase is known to partly dephosphorylate phosphodextrins (14) to the extent of 75%; the residual 25% remains resistant to further phosphorolysis, presumably due to steric hindrance (15). Potato starch β-dextrin which was treated with alkaline-phosphatase in vitro permitted the larvae to grow slowly and pupate. This observation indicates that the nutritional value of the indigestible phosphodextrin is improved by partial dephosphorylation.

The product of predigestion of potato starch β-dextrin with human salivary α-amylase adequately supports larval development of *Tribolium*.[4] This observation indicates that the esterified phosphate per se is not responsible for the inability of *Tribolium* to utilize the β-dextrin, but that its presence in the inner chains of the intact phosphodextrin imparts resistance to amylolysis in this case.

Hydrolysis of starch subfractions. The hydrolysis of starch subfractions by *Tribolium* amylase and by salivary α-amylase during a 5-hour incubation period is presented in figures 1–3. Their action patterns differ in many respects. Maltose and maltotriose (G_2, G_3) accumulate in all 3 cases of digestion by salivary α-amylase, with only traces of glucose (G_1) appearing. Higher dextrins do not accumulate at this stage. The presence of esterified phosphate in the β-dextrin of potato starch apparently does not interfere in the first stage of hydrolysis by human salivary α-amylase (compare figs. 1a and 2a), although during prolonged action for several days, hydrolysis enters a second stage in which the effect of phosphate has been reported (15). A marked difference is noted in the products of hydrolysis of potato starch β-dextrin and of commercial amylopectin β-dextrin by *Tribolium* amylase: In the former, which is not utilized by larvae for development, little G_4–G_6 accumulates (fig. 2b), whereas in the latter, with which larval development is optimal (1), a definite accumulation of G_4–G_6 is observed (fig. 1b). The accumulation of these higher dextrins presumably indicates a greater degree of inner-chain hydrolysis. The action pattern of *Tribolium* amylase on dephosphorylated β-dextrin of potato starch differs from its action pattern on untreated β-dextrin in the accumulation of G_3 and of higher dextrins, and in the absence of G_1 (fig. 3b).

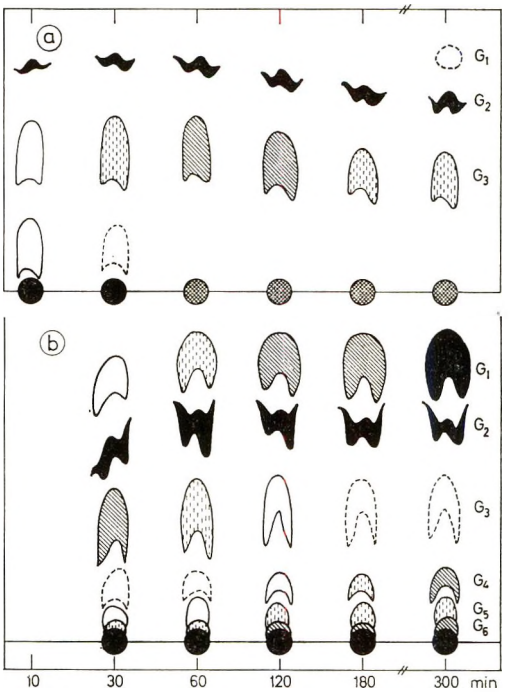

Fig. 1 Diagrammatic presentation of the comparative action patterns of human salivary α-amylase (a) and *Tribolium* amylase (b) on β-dextrin of commercial amylopectin. The degree of crosshatching represents color intensity.

A highly purified α-amylase inhibitor from wheat (16) completely inhibits *Tribolium* amylatic activity. The unusual action patterns encountered cannot, therefore, be attributed to the coincident action of auxiliary hydrolytic enzymes. *Tribolium* amylase is intermediate in its action between salivary α-amylase, and seed α-amylases, which initially produce G_6–G_8 (17).

[4] This product was submitted to TLC and shown to be representative (in respect to oligosaccharide content) of a digestion product of potato starch β-dextrin by salivary α-amylase

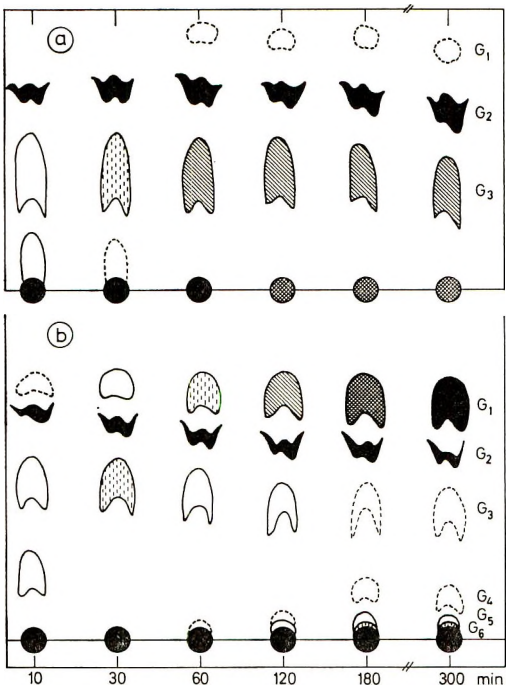

Fig. 2 Diagrammatic presentation of the comparative action patterns of human salivary α-amylase (a) and *Tribolium* amylase (b) on β-dextrin of potato starch. The degree of crosshatching represents color intensity.

Larval midgut phosphatase activity. The pH dependence of *Tribolium* phosphatase is illustrated in figure 4. The pH optimum of 7.0 rules out the possibility of attributing this activity to wheat germ acid phosphatase, whose pH optimum is 5.8 (18), from residues of the whole wheat diet with which the larvae were reared. The level of phosphatase activity, under optimal conditions, in late-instar larval midguts, is illustrated in figure 5. It is interesting to compare these results with the reported presence of both acid and alkaline phosphatase activity in *T. confusum* Duval. larvae (19, 20). The assays on *T. confusum* were made on total homogenates of whole larvae, in contrast with the present determinations on *T. castaneum*, which were made on midgut homogenates.

It is difficult to assess the in vivo function and actual activity of the larval midgut phosphatase from the growth experiments conducted. The esterified phosphate in the β-dextrin of potato starch was not effectively hydrolyzed in vivo, since pre-treatment in vitro of the β-dextrin with calf-intestinal alkaline phosphatase improved its subsequent digestibility (table

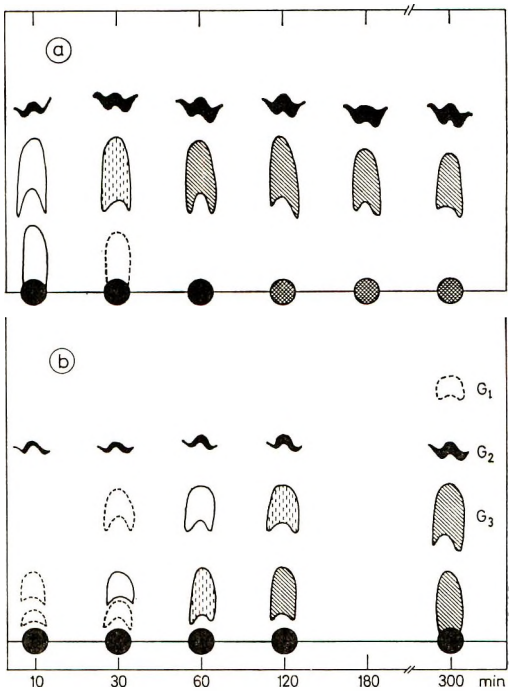

Fig. 3 Diagrammatic presentation of the comparative action patterns of human salivary α-amylase and *Tribolium* amylase (b) on phosphatase-treated β-dextrin of potato starch. The degree of crosshatching represents color intensity.

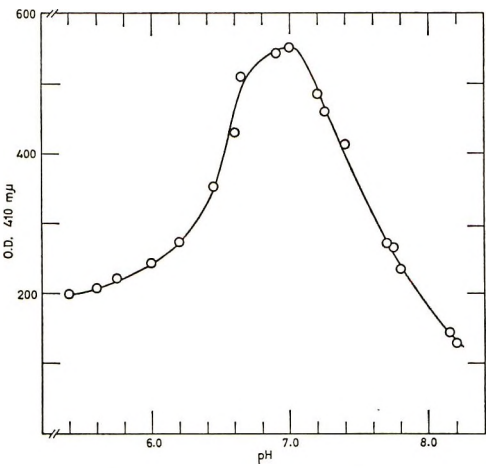

Fig. 4 The pH dependence of *Tribolium* phosphatase activity.

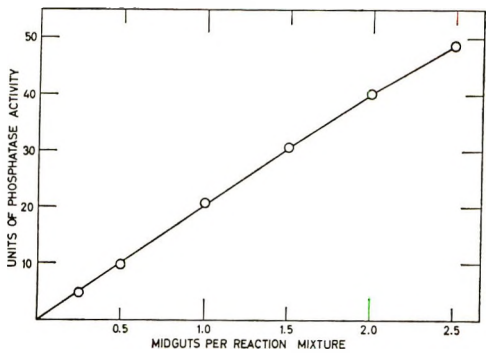

Fig. 5 Effect of enzyme concentration on phosphatase activity of *Tribolium* larvae.

2). This might be explained by assuming that we are dealing with an intracellular enzyme which is not excreted into the lumen of the larval midgut and is not a digestive enzyme in the strict sense. An alternative explanation, however, might be that the inorganic phosphate present in the salt mixture component of the basic diet (table 1) inhibits the midgut phosphatase. This might explain the absence of any growth improvement in the alkaline-phosphatase-supplemented diet (table 2). The main point to be stressed is that even when the potato starch β-dextrin is partially dephosphorylated, it is not utilized by *Tribolium* larvae as well as β-dextrin from other sources, and this is presumably because of the specific mode of action of the larval amylase, which differs markedly from amylases of higher animals.

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Glucose Milliequivalents Eaten by the Neonatal Pig and Cessation of Intestinal Absorption of Large Molecules (Closure) [1,2]

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ABSTRACT Neonates of some species, e.g., piglets, are able to absorb large molecules through their intestines for approximately the first 36 hours of their life. The time when the piglet ceased absorbing large molecules (closure) was a function of feeding regimen. The purpose of the present experiments was to define further the means whereby feeding accelerated closure. It was learned that the absorbing capacity of the neonatal pig was diminished by feeding it water solutions of one chemical molecular species — for example, glucose. Piglets that ate more than 300 mEq of glucose within an 18- to 24-hour period were unable to absorb the 40-ml test dose of chicken egg protein given *per os*. Piglets fed solutions of galactose, xylose, sucrose, and lactose reacted similarly. Salt solutions, glycine and lecithin were not effective. The closure response to glucose was independent of the total volume fed and the concentration of glucose. It was not possible to effect closure in piglets in less than 12 hours, but from then on closure was independent of time and dependent on the number of molecules of glucose consumed. Reversal of closure was not detected.

Previously (1), closure (the period after which the neonate can no longer absorb large molecules through the gut) was shown to be a function of the diet; namely, the volume of sow's or cow's colostrum consumed by the piglet. Further work (2) with purified dietary factors demonstrated the association of closure activity with protein-, fat-free cow's colostrum, dialysates of cow's colostrum and dialysates of non-fat milk solids. Salt solutions similar in composition to the salts in cow's milk plus either 3% glucose or 2% lactose did not yield closure under our test conditions.

Following this, experiments were designed to delineate further these observations using dietary regimens that had as a basal diet a milk salt solution with 3% glucose. To this presumed negative basal diet other components were added and fed to the piglet for the first 18 hours of his life. To determine whether the diet promoted closure at 24 hours of age, the piglet was given *per os* a test dose of a water solution of polyvinylpyrrolidone [3] (PVP) as an absorption marker. Six hours later the piglet's blood was analyzed for PVP. Equivocal results were obtained in a number of expermients that tested the addition of various fats, fatty acids, and phospholipids.

The equivocal results may, in part, have stemmed from the system used for detecting absorption and from the possible presence of some closure activity in our basal salt-glucose diet. Subsequently it was shown that water solutions of high molecular weight materials may not be optimal absorption markers since they lack natural components that accelerate and promote rapid absorption by the neonatal gut (3). To circumvent these possible difficulties, in the experiments reported herein homogenized whole egg was given as the absorption marker and a serological precipitin test was used for the detection of egg protein in the piglet's blood. In addition, rather than adding material to be tested for closure activity to the salt-glucose basal diet, water solutions of single chemi-

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[2] Presented in part at Symposium, "Development of Metabolism as Related to Nutrition," Prague, Czechoslovakia, January 1966.

[3] Average molecular weight of 40,000. Antara Chemicals, New York.

cal molecular species were fed. Since glucose was suspect, this was the first dietary component tested for activity.

MATERIAL AND METHODS

Experimental animals. Piglets farrowed in an isolation unit were caught at birth in sterile towels, transferred to another isolation unit and caged individually. The basic procedure was to feed the piglet from a pan up to 600 ml of a randomly assigned diet within a 24-hour period. The first feeding occurred within 4 hours after the last pig was born. Thereafter, piglets were fed at 6–hour intervals. Thus, the second feeding came 6 hours after the first; the third feeding, 12 hours after the first; and the fourth and final feeding, 18 hours after the first.

A total of 75 pigs from 11 litters was used.

Closure testing system. Six hours after the last feeding, piglets were given 40 ml of homogenized chicken eggs by stomach tube to test for their absorbing capacity. Six to 8 hours later they were bled and their serum analyzed by microimmunoelectrophoresis (4, 5) for the presence of egg white proteins. Positive control pigs absorbed about 10% of the protein and negative pigs (closed) less than 0.2%.

Diets. All the diets except the salt diet were water solutions of one molecular species. In addition to glucose, piglets were fed 600 ml of either 666 mM galactose, 666 mM xylose, 666 mM glycine, 333 mM lactose or 333 mM of sucrose within a 24-hour period. It was difficult to compound a salt diet that had sufficient milliequivalents per liter of salt ions for testing and yet was not toxic for the piglets. Thus, it was necessary to add small amounts of glucose to increase the total milliequivalents per liter to a desirable level. The first salt diet tested contained 113 mEq/liter of Na^+, 149 of K^+, 145 of Cl^-, 183 of PO_4^{3-} and 166 of glucose. This was superseded by another that was found to be more palatable to the piglets. This diet contained: 69 mEq/liter of Na^+, 94 of K^+, 90 of Cl^-, 117 of PO_4^{3-} and 266 of glucose.

To plot the data on a comparable basis, each molecular or ionic species tested was expressed as milliequivalents eaten by the piglet. The potential closure capacity of 1 mEq of salt ion was assumed to be equivalent to 1 mEq of glucose, and 1 mM of lactose or sucrose was equivalent to 2 mEq of glucose.

Three different types of feeding regimens were conducted to determine the closure capacity of glucose. In the first type, the milliequivalent of glucose the piglet ate in a 24-hour period was varied by varying the concentration of glucose in a 600-ml volume. For example, some piglets were fed a total of 450 mEq in 600 ml, others 400 mEq in 600 ml, etc. In the second type, the milliequivalents were varied by keeping the concentration of glucose in the diet constant and varying the volume the piglet ate over a 24-hour period. For example, some piglets were fed 13.5% glucose in 300 ml (225 mEq); others 13.5% glucose in 600 ml (450 mEq); some pigs ate 300 ml of 10% glucose (175 mEq); and others 600 ml of 10% glucose (350 mEq), etc. In the last type, piglets were allowed to eat paired milliequivalents of glucose within varying time periods. For example, some piglets were given 350 mEq of glucose within 12 hours, whereas others were given the 350 mEq over either an 18- or 24-hour period.

RESULTS

Milliequivalents glucose eaten. Figure 1 shows a compilation of data from experi-

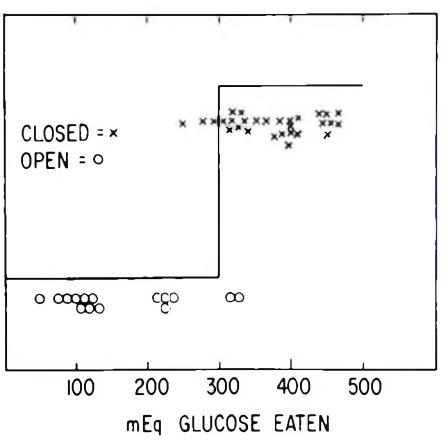

Fig. 1 Effect of milliequivalent of glucose eaten on closure (inability to absorb egg protein 6 hours after the last feeding). Piglets fed either 600 ml of varying concentrations of glucose or varying volumes of glucose within an 18- to 24-hour period. Each X or O = 1 piglet.

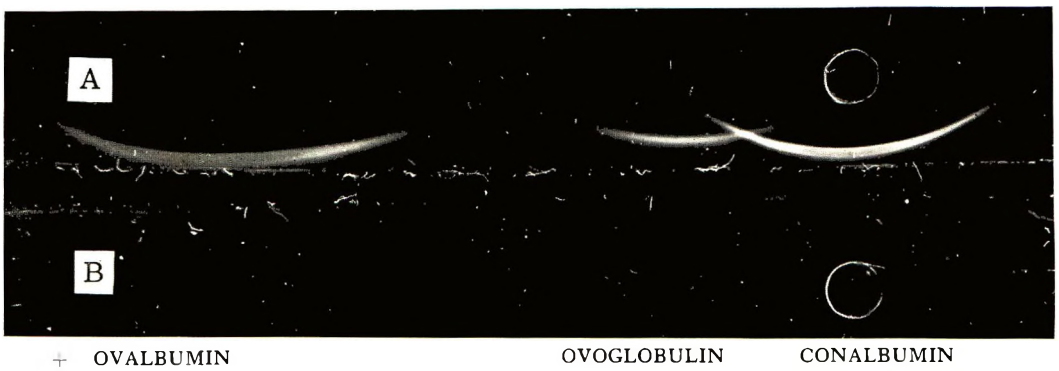

Fig. 2 Agar microimmunoelectrophoresis of 0.004 ml of serum from 2 piglets given *per os* 40 ml whole egg at 24 hours. *A*, Top hole, 0.004 ml of serum from piglet eating for 24 hours a total of 80 mEq glucose in 600 ml. *B*, Bottom hole, 0.004 ml serum from piglet eating for 24 hours a total of 400 mEq glucose in 600 ml. Center slit, 0.04 ml of rabbit anti-egg white serum. Piglets were bled 6 hours after receiving egg. + indicates direction of electrophoretic migration in veronal buffer, pH 8.6; 0.05 ionic strength.

ments in which the total milliequivalents of glucose the piglet ate in an 18- to 24-hour period were varied either by changing the concentration of glucose or the volume of the diet. The data indicate that the absorbing capacity of the piglet was diminished after it had eaten around 300 mEq of glucose. Those piglets eating more than 300 mEq did not absorb the test solution of egg protein according to the standards of our test (absorbed less than 0.2% of the egg protein given *per os*).

The kinds of results obtained with the microimmunoelectrophoretic testing system are shown in figure 2. In figure 2A, serum is analyzed from a piglet that ate a total of 80 mEq of glucose in 600 ml within 24 hours. At 24 hours it was given 40 ml of whole homogenized egg and was bled 6 hours later. Note the presence of egg protein in its blood. A littermate eating 400 mEq of glucose in 600 ml within 24 hours and tested similarly had no detectable egg protein in its blood and the piglet was classified as closed (fig. 2B).

Volume and/or concentration (milliequivalents/liter) of glucose. The data in figure 3A were compiled to illustrate that it is the total milliequivalents of glucose the piglet eats that is important for closure rather than the concentration per se of the glucose in the diet or the volume consumed. Here, piglets were fed varying milliequivalents of glucose by keeping the concentration of glucose constant and varying the volume of the diet given to the piglet. For example, some piglets were fed either 300 or 600 ml of 10% glucose; others 300 or 600 ml of 13.5% glucose, etc. Figure 3A shows that closure is independent of the concentration and volume of glucose since closure begins when piglets eat approximately 300 mEq regardless of whether the glucose is contained in 300 to 450 ml (high glucose concentration) or in 500 to 600 ml (low glucose concentration).

Time. The data in figure 3B came from piglets that were fed the same milliequiv-

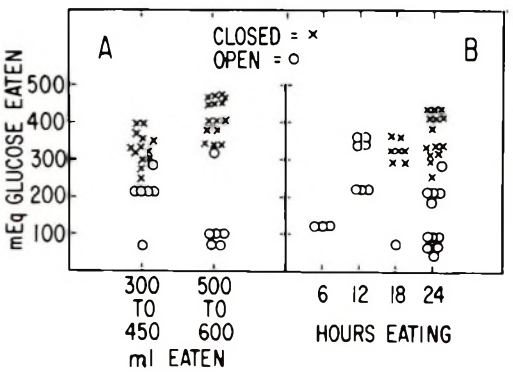

Fig. 3 Effect of volume (concentration of glucose) and length of feeding time on closure (inability to absorb egg protein 6 hours after last feeding). *A*, Piglets were fed varying volumes of glucose within 18 to 24 hours. *B*, Piglets were fed paired amounts of glucose within varying time periods. Each X or O = 1 piglet.

alents of glucose within varying periods of time. Figure 3B illustrates that it is not possible to close piglets within 12 hours since some piglets ate what was considered a closing amount of glucose (350 mEq) within 12 hours and they were not closed. Note that piglets fed approximately the same milliequivalents but within an 18- or 24-hour period were closed.

Specificity of closure molecule. From the results in figure 4, it is doubted that closure activity is specific with glucose since galactose, lactose, xylose and sucrose appear to have activity. If activity is present in the electrolyte-glucose solution and glycine, it appears to be of lower order. Glycine is difficult to test since piglets reluctantly ate no more than 300 mEq within 24 hours. Pure salt solutions also are difficult to test since piglets eating much more than 300 mEq salt have symptoms of salt toxicity (hypersensitive to touch, and death). Therefore, in the salt diet, to bring the total equivalents up to presumed closure quantities (300 mEq or more), this necessitated the addition of less than closure equivalents of glucose to the salt solution.

One per cent lecithin [4] also was tested in 3 piglets (glucose equivalents unknown) and did not yield closure.

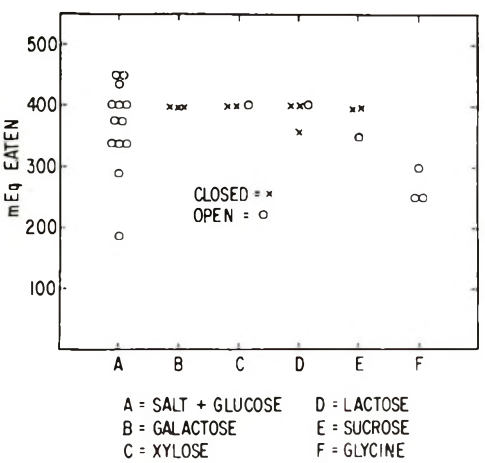

Fig. 4 Effect of molecules other than glucose on closure (inability to absorb egg protein 6 hours after the last feeding). 1 mEq salt ion = 1 mEq glucose; 1 mM galactose, xylose or glycine = 1 mEq glucose; 1 mM sucrose or lactose = 2 mEq glucose. Each X or O = 1 piglet.

Attempt to detect a reversal of closure. Six piglets from 2 different litters were fed 400 mEq of glucose within 18 hours and tested with 40 ml of egg. Six hours later they were retested with an identical dose of egg. These piglets were closed on both occasions. In another experiment, 9 piglets were fed 400 mEq of glucose within 24 hours; and instead of testing as usual at 24 hours (6 hours after the last feeding), the testing time was delayed so that 3 pigs were tested at 10, 3 pigs at 14, and 3 pigs at 20 hours after the last feeding. All these pigs remained closed; and thus, within our experimental design, no reversal was detected.

DISCUSSION

These results reaffirm and amplify results of previous studies; namely, it is possible by feeding neonatal piglets to show a decrease in absorption capacity toward large molecules (closure) in comparison with starved piglets (1,2,6). In this report it was shown further that the capacity to absorb large molecules can be diminished simply by feeding piglets increasing concentrations of a single molecular species. In our test, piglets eating about 300 mEq of glucose within an 18- to 24-hour period were closed. It is unlikely that this action is strictly specific for glucose since lactose, xylose, galactose and sucrose also appear to be able to act in a similar manner. And, furthermore, if we are studying the same closure phenomenon as it occurs in nature, glucose per se cannot be important since it is a minor part of the natural diet of the newborn pig. However, a comparison of the results of sugars with salt solutions, glycine and lecithin indicates that some molecules may be more efficient than others.

Pinocytosis (absorption by folding interiorizing of the surface membrane) presumably is the mechanism whereby the immature intestinal epithelium of newborn piglets absorbs large molecules (7). In previous work, this absorption process was shown to be qualitatively non-specific since antigenically different egg proteins, bovine proteins, and isoenzymes of lactic

[4] 90% pure animal lecithin. Nutritional Biochemicals Corporation, Cleveland.

dehydrogenase and different kinds of large molecules such as polyvinylpolymers (PVP) were absorbed by the neonate (8,9). The data in the present report also indicate another kind of non-specificity associated with the immature piglet's epithelium. Here, the capacity of a diet to diminish absorption appears to be more dependent on the numbers of molecules rather than the kinds of molecules.

If pinocytosis proceeds by the interiorization of the surface membrane, it seems probable that the capacity to absorb would be limited by the luminal surface of the epithelial cell. Thus, it might be proposed that closure (inhibited pinocytosis) occurs when critical surface area is expended. It appears possible that many different kinds of large and small molecules could stimulate the surface membrane into a dance of indentation leading to exhaustion of surface (or closure) some 300 mEq later. If large molecules are present during this dance, they could be adsorbed and then absorbed; if not, the membrane becomes used anyway. Thus, absorption of large molecules and closure can be independent phenomena as they were shown to be previously (2). Furthermore, the time required for closure (the speed of discharge or use of the surface membrane) appears to be directly related to the number of molecules confronting the cell.

To summarize, then, perhaps absorption by neonatal gut of large molecules can be diminished by non-specific stimulation of pinocytosis leading to a loss of or unavailability of surface membrane for invagination. Such exhaustion in the piglet can cocur within 18 hours after feeding sufficient numbers of a stimulant.

ACKNOWLEDGMENT

The author gratefully acknowledges the technical assistance of Mrs. Carolyn W. Broughton.

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Accentuation of Essential Fatty Acid Deficiency by Dietary Tri-o-cresyl Phosphate

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ABSTRACT Dietary addition of tri-o-cresyl phosphate (TCP) to an EFA-free 18% casein diet accentuated the deficiency and decreased the time of onset of dermal lesions in rats. Dietary intake of TCP caused increases in dermal scores and eicosatrienoic acid (E) and decreases in arachidonic acid (A) in liver lipids of both the deficient and the control groups. In carcass lipid, however, dietary additions of TCP caused decreases in both eicosatrienoic and arachidonic acids of the deficient groups. TCP depressed growth of the deficient and control rats; however, the depression was greatest in the deficient animals. Dietary TCP accentuated the decrease in oxidative phosphorylation noted in an uncomplicated EFA deficiency. Liver cholesterol increased in EFA-deficient rats, and this was accentuated by dietary TCP. Values for total lipids of liver, heart and carcass are also reported.

Burr and Burr (1) discovered that certain fatty acids are essential in the diet. The biochemical role of these essential fatty acids (EFA), however, has attracted intensive research interest only during the last few years. Various metabolic approaches, with the rat as the experimental animal, have been used to elucidate the specific role of essential fatty acids in living organisms. Several investigators have attempted to accelerate the onset of signs of EFA deficiency in rats.

Because of increased mobilization and catabolism of essential fatty acids in rats with diabetes, Peifer and Holman (2) induced diabetes in rats as a means of increasing the rate of EFA depletion. On the basis of dermal scores and growth differences they concluded that diabetes had an accelerating effect on EFA deficiency in the rat. Dietary cholesterol has been used to accelerate the onset of EFA deficiency. Bromer and Day [1] observed that male rats fed diets containing 2% cholesterol and 3% hydrogenated peanut oil showed signs of EFA deficiency after 7 weeks as compared with 12 weeks when fed a diet which did not contain cholesterol. Peifer and Holman [2] subsequently showed that male rats fed 1% cholesterol and 1% hydrogenated coconut oil developed EFA deficiency within one month. More recently, Holman and Peifer (3) reported that the inclusion of 1% cholesterol in an EFA-free diet accelerates the appearance of dermal signs of EFA deficiency. The acceleration was also indicated by an increase in ratios of trienoic to tetraenoic acid in liver fatty acids.

Hove (4) reported that several compounds including tri-o-cresyl phosphate (TCP) caused a decrease in the liver content of vitamin E and vitamin A in the rat. Liver, brain and carcass lipids from rats fed TCP in the absence of vitamin E showed increases in the concentration of conjugated dienes and decreases in apparent arachidonic acid levels. He advanced the hypothesis that the mechanism of action of TCP is related to its generalized oxidative action in unsaturated lipid systems.

We decided to test the capacity of TCP to accelerate EFA deficiency in the rat. These accelerating effects of TCP on EFA deficiency were studied in terms of several of the more common indexes of EFA deficiency; dermal lesions, ratios of eicosatrienoic to arachidonic (tetraenoic) acid, liver cholesterol, and oxidative phosphorylation ratios in liver mitochondria. The

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TABLE 1

Effect of tri-o-cresyl phosphate (TCP) on dermal scores and weight gains of essential fatty acid-deficient and control rats

Dietary addition		Dermal scores		Daily wt gain
Corn oil	TCP	4 Weeks	8 Weeks	
%	%			g
0	0	0.8 ± 0.09 [1]	1.8 ± 0.16	4.9 ± 0.17
0	0.05	1.5 ± 0.15	2.3 ± 0.12	2.8 ± 0.06
0	0.10	2.2 ± 0.18	3.4 ± 0.12	2.0 ± 0.10
2	0	0	0	4.3 ± 0.14
2	0.05	0	0	4.1 ± 0.10
2	0.10	0	0	2.2 ± 0.13

[1] Mean ± SE; visual grading of 0–4; 4 represents complete scaling of paws and necrosis of tail; 10 rats/group.

effect of TCP on heart, liver and carcass fat will also be described.

EXPERIMENTAL

Male weanling rats of the Holtzman strain, 21 days old and weighing 45 to 50 g, were used in these studies. The animals were housed individually in raised wire-bottom cages at a constant room temperature of 23 ± 1°. Diets and tap water were available ad libitum. Weight data and observation of the general physical appearance were recorded weekly. The dermal scores represent the average visual grading of 0 to 4, where a score of 4 represents complete scaling of all 4 feet and necrosis of the entire tail.

The basal diet (EFA-free) contained: (in per cent) sucrose, 72; casein (vitamin-free), 15; medium-chain triglycerides,[3] 5; salt mix (Jones-Foster),[4] 4; and vitamin mix (A and E-free) 4. The vitamin mix was prepared in glucose monohydrate[5] to supply: (in μg/g of diet) thiamine, 5; riboflavin, 5; pyridoxine, 5; Ca pantothenate, 30; niacin, 30; menadione, 2; folic acid, 1; vitamin B_{12}, 0.1; biotin, 0.1; inositol, 200; and choline, 2000. To this diet were added 5 mg of retinyl acetate and 200 mg of *dl*-α-tocopheryl acetate/kg.

Ten weanling rats were used in each of 6 groups in which all additions were made to the basal diet. Group 1 was fed the basal diet; group 2, 0.05% TCP; group 3, 0.1% TCP; group 4, 2% corn oil at the expense of 2% MCT (control); group 5, control plus 0.05% TCP; and group 6, control plus 0.1% TCP.

Respiration and oxidative phosphorylation experiments were carried out as described elsewhere (5). The methods used in total fat analysis of heart, liver and carcass tissues have been reported previously (6). Tissue proteins (N × 6.25) were determined by the micro-Kjeldahl method. Total cholesterol was determined by the digitonide precipitation method of Sperry and Webb (7) and vitamin A by the method of Ames et al. (8). The fatty acid profiles were determined on aliquots of the methyl esters. Methanolysis was carried out by using 3% H_2SO_4 in absolute methanol. The fatty acid methyl esters were chromatographed in an Aerograph 600 C gas chromatograph, equipped with a hydrogen flame detector and a 152.4-cm × 0.31-cm copper column of 19% EGS on 60–80 mesh Gas-Chrom P.[6] Identification was established by comparison with standards[7] or carbon number (9), or both.

RESULTS

Dermal lesions and weight gain. Skin lesions typical of essential fatty acid deficiency (1, 10) began to appear as early as 4 weeks, and were much more severe among the rats receiving the EFA-deficient diet containing TCP (table 1). Among

[3] Medium-chain tryglycerides (MCT) consist of resynthesized triglycerides from fatty acids of 8–14 carbon chain length, hydrolyzed from coconut oil and purified. Obtained from the Mead Johnson Research Center, Evansville, Indiana.

[4] Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutr., 24: 245.

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

[6] Pretested packing and methyl ester standards, qualitative and quantitative, were obtained from Applied Science Laboratories, State College, Pennsylvania.

[7] See footnote 6.

the rats receiving the EFA-deficient diet supplemented with 0.1% TCP, the characteristic signs of EFA deficiency were accompanied by a moist, colorless exudate appearing on swollen paws and lower legs. The effect of 0.05% TCP was similar to that at the higher level but was less severe. None of the typical skin lesions of EFA deficiency were observed among the rats receiving 2% corn oil in their diets, with or without the addition of TCP.

Essential fatty acid deficiency resulting from ingesting a diet containing MCT as the only fat source caused no apparent differences in weight gains compared with controls (table 1). However, growth rates were depressed due to TCP. This effect of TCP is more pronounced in the EFA-deficient rats than in the rats fed corn oil, as shown by the weight gain data in table 1.

Eicosatrienoic to arachidonic acid ratios. The ratios of trienoic: tetraenoic acids are excellent indicators of the EFA deficiency status of the rat (11). The levels of eicosatrienoic (E) and arachidonic (tetraenoic) (A) acids and the E/A ratios of heart, liver and carcass are shown in table 2. In all tissues the E/A ratios increase with increasing levels of TCP in the EFA-deficient diets as well as the control.

Oxidative phosphorylation ratios of liver mitochondria. An uncoupling of oxidation from phosphorylation was demonstrated in the mitochondrial fraction of the whole homogenate of liver from EFA-deficient rats. Data in table 3 summarize a typical experiment with respect to oxidative phosphorylation with β-hydroxybutyrate as substrate. The inorganic phosphorus (Pi) uptake was low in the EFA-deficient rat compared with that of the control (2% corn oil). Addition of TCP to the EFA-deficient diet further depressed Pi uptake with little or no change in O_2 consumption. This resulted in depressed P/O ratios with increasing levels of dietary TCP. When these same levels of TCP were added to the control diet, the P/O ratios did not change; however, Pi as well as O_2 uptake at the 0.1% level decreased slightly.

Liver weight, cholesterol, and vitamin A. Animals fed an EFA-deficient diet

TABLE 2

Effect of tri-o-cresyl phosphate (TCP) on eicosatrienoic acid (E) to arachidonic (A) acid ratios in liver, heart and carcass of essential fatty acid-deficient and control rats

Dietary addition		Heart fatty acids			Liver fatty acids			Carcass fatty acids		
Corn oil	TCP	E	A	E/A	E	A	E/A	E	A	E/A
%	%	%	%		%	%		%	%	
0	0	10.6 ± 0.85 [1]	9.4 ± 0.88	1.13	3.9 ± 0.23	14.7 ± 0.60	0.27	5.5 ± 0.63	4.0 ± 0.42	1.38
0	0.05	12.1 ± 0.32	8.5 ± 0.69	1.42	7.7 ± 0.73	6.9 ± 0.85	1.12	5.2 ± 0.75	3.5 ± 0.95	1.49
0	0.10	14.4 ± 0.44	8.0 ± 0.90	1.80	12.4 ± 0.45	6.7 ± 0.45	1.85	2.9 ± 0.56	1.9 ± 0.49	1.53
2	0	0.9 ± 0.05	23.2 ± 1.00	0.04	1.6 ± 0.23	19.4 ± 0.60	0.08	0.5 ± 0.12	6.6 ± 1.72	0.08
2	0.05	1.3 ± 0.15	15.5 ± 2.90	0.08	2.0 ± 0.08	17.3 ± 0.08	0.12	0.7 ± 0.08	5.2 ± 1.56	0.13
2	0.10	2.3 ± 0.32	9.7 ± 0.75	0.24	2.3 ± 0.48	14.9 ± 0.33	0.15	1.1 ± 0.03	3.7 ± 0.96	0.30

[1] Mean ± SE (as % methyl ester); 4 rats from each group; fed diet 8 weeks.

TABLE 3

Effect of tri-o-cresyl phosphate (TCP) on oxidative phosphorylation in liver mitochondria from essential fatty acid-deficient and control rats

Dietary addition		Δ O_2	Δ Pi	P/O ratios
Corn oil	TCP			
%	%	*μatoms/mg* N^2	*μmoles/mg* N^2	
0	0	4.3	9.6	2.2 ± 0.08 [1]
0	0.05	3.7	7.4	2.0 ± 0.24
0	0.10	3.5	6.3	1.8 ± 0.05
2	0	4.1	12.2	3.0 ± 0.10
2	0.05	4.2	12.6	3.0 ± 0.23
2	0.10	3.1	9.0	2.8 ± 0.30

[1] Mean ± SE; β-hydroxybutyrate as substrate; 4 rats from each group; fed diet 8 weeks.
[2] N represents liver mitochondria nitrogen determined by micro-Kjeldahl method.

supplemented with TCP showed increases in liver weights (expressed as % of body weight) with increasing levels of TCP (table 4). The effect of TCP on liver weights of the control rats was similar to that on the EFA-deficient animals.

Liver cholesterol levels of rats ingesting the EFA-deficient diets increased in comparison with those of rats receiving corn oil (table 5). When the data are expressed as milligrams per gram of liver (wet weight) the addition of TCP has no effect in either group. When the data are expressed in terms of 100 g of body weight of the animal, however, increasing levels of TCP caused an increase in the amount of cholesterol in the livers of the rats fed the EFA-deficient diet but caused no increase in the controls.

Liver vitamin A levels appear to decrease with increasing levels of TCP (table 5); this apparent decrease probably reflects only the changes in liver size or vitamin A intake.

Total heart, liver and carcass fat. In table 4 are presented the mean total fat values (wet weight) for heart, liver and carcass. Liver fat values in the EFA-deficient animals are higher than those of controls at each level of TCP. TCP had little or no effect on percentage of fat in the heart or carcass, irrespective of the diet. When the fat values are expressed in terms of body weight, however, the fat content of heart and liver gradually increases with increasing levels of TCP in both the EFA-deficient and the control groups; carcass fat does not increase. There is no evidence of change in heart size as a response to TCP with or without corn oil in the diet.

DISCUSSION

In the present studies dermal scores were proportional to time for the first 8 weeks (table 1); these data are in agreement with those of Holman and Peifer (3). We further found that the effect of TCP on group mean dermal scores paralleled heart, liver and carcass eicosatrienoic acid concentrations (with the one exception of carcass fatty acid, table 2), as well as the ratio of eicosatrienoic to arachidonic acids in rats ingesting the EFA-deficient diet.

The development of EFA deficiency is accompanied by a relative increase in trienoic acids and a relative decrease in tetraenoic acids (14). In our experiment the increase in eicosatrienoic acid in heart and liver (with one exception) with a concomitant decrease in arachidonic acid in liver (table 2) was one of the more striking effects in tissues from rats fed TCP in the basal as well as the control diets; these effects indicated an intensified EFA deficiency. Even though the ratios for heart, liver and carcass fatty acid from rats on a corn oil regimen were less than 0.4, the limit of this criterion for EFA deficiency (11), the changes in the ratios were related to TCP concentrations.

In disagreement with a number of investigators (12, 15, 16) who have reported differences in weight gain of various species of animals fed EFA-free diets compared with controls, we found no

TABLE 4

Effect of tri-o-cresyl phosphate (TCP) on heart and liver weights; heart, liver and carcass fat in essential fatty acid-deficient and control rats

Dietary addition		Heart wt	Liver wt	Total fat (wet wt)			Heart fat	Liver fat	Carcass fat
				Heart	Liver	Carcass			
Corn oil	TCP								
%	%	% body wt	% body wt	%	%	%	mg/100 g body wt	mg/100 g body wt	mg/100 g body wt
0	0	0.3 ± 0.02 [1]	3.8 ± 0.07	3.5 ± 0.23	6.7 ± 0.25	13.9 ± 0.56	10.3	252.5	12.2
0	0.05	0.3 ± 0.01	4.5 ± 0.21	3.9 ± 0.33	5.8 ± 0.17	12.6 ± 0.54	11.6	260.0	11.1
0	0.10	0.4 ± 0.02	5.4 ± 0.22	3.1 ± 0.40	5.3 ± 0.07	13.0 ± 0.40	12.9	278.0	11.4
2	0	0.3 ± 0.03	4.0 ± 0.05	3.2 ± 0.13	4.3 ± 0.29	12.9 ± 0.44	9.4	173.6	11.4
2	0.05	0.3 ± 0.01	4.3 ± 0.11	4.0 ± 0.47	5.1 ± 0.28	12.9 ± 0.33	11.4	238.6	11.1
2	0.10	0.4 ± 0.01	5.6 ± 0.21	4.1 ± 0.24	4.3 ± 0.22	12.1 ± 0.68	13.3	221.4	9.3

[1] Mean ± SE; 10 rats/group; fed diet 8 weeks.

weight gain differences among rats that did not receive TCP and that ingested MCT as their only fat source. These observations are in agreement with those of Sewell and Miller (13), who report no weight gain differences between swine fed an EFA-free diet and those fed the control diet. It thus appears that growth rate is not always a reliable criterion in ascertaining EFA deficiency in the rat.

Several reports of long standing and some of more recent origin have amply demonstrated that EFA deficiency causes an uncoupling of oxidation from phosphorylation (17–20). Our results support this view. Uncoupling appears to be specifically related to the absence of essential fatty acids rather than to fats in general. This distinction was noted by Smith and DeLuca (21), who reported that liver homogenates from EFA-deficient rats oxidized Krebs' cycle acids at a much higher rate than did homogenates from controls; this could be the result of uncoupled oxidative phosphorylation in homogenates from EFA-deficient rats. The effect of TCP on P/O ratios of liver mitochondria from EFA-deficient rats was inversely proportional to its effect on dermal scores, eicosatrienoic acid levels and E/A ratios, indicating an accentuation of EFA deficiency by TCP.

From the data in tables 4 and 5 cholesterol and total lipids apparently accumulated in the livers of the EFA-deficient rats. An increase in the concentration of cholesterol esters has been observed previously in the livers of young EFA-deficient rats by Klein (22) and Norby (12) and in the adult EFA-deficient rat by Alfin-Slater et al. (23). The reasons for this accumulation of cholesterol and total lipids are not clear. According to Mukherjee and Alfin-Slater (24) the hepatic metabolism of cholesterol is decreased in EFA-deficient rats, thereby resulting in the observed fat accumulation. The increase of hepatic lipids in the EFA-deficient rat with increasing levels of TCP may also indicate that the metabolism of cholesterol and other lipids is in some way related to the overall polyenoic acid pattern in this tissue.

Hands et al. (25) have reported that the visual acuity of EFA-deficient rats is

TABLE 5

Effect of tri-o-cresyl phosphate (TCP) on liver cholesterol and vitamin A of essential fatty acid-deficient and control rats

Dietary addition		Total cholesterol		Vitamin A
Corn oil	TCP			
%	%	*mg/g (wet wt)*	*mg/100 g body wt*	*μg/g (wet wt)*
0	0	3.3 ± 0.13 [1]	12.3	374 ± 18
0	0.05	3.0 ± 0.12	13.7	334 ± 15
0	0.10	3.1 ± 0.09	16.4	248 ± 19
2	0	2.7 ± 0.05	11.1	255 ± 13
2	0.05	2.7 ± 0.12	11.9	257 ± 15
2	0.10	2.1 ± 0.05	11.6	135 ± 19

[1] Mean ± SE; 5 rats from each group; fed diet 8 weeks.

impaired and that this impairment resulted from an induced vitamin A deficiency. We noted no vitamin A deficiencies in any of our EFA-deficient rats. Liver storage appeared to decrease in the presence of TCP in both EFA-deficient and control groups, but this change may reflect only the change in liver size or food consumption owing to TCP intake; nevertheless, liver stores of vitamin A were high enough to rule out a secondary vitamin A deficiency.

Numerous reports have shown that TCP accentuates the requirement for vitamin E; these reports have been reviewed by Hove (26).

Our results show that dietary TCP can be a stress factor accentuating or accelerating EFA deficiency in the rat, possibly through a mechanism associated with the generalized oxidative activities of TCP in unsaturated lipid systems (4).

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Renal Lipid Composition of Choline-deficient Rats [1]

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ABSTRACT Three groups of weanling male rats of the Sprague-Dawley strain were used in a study of the changes in lipid composition between normal kidneys and severely hemorrhagic kidneys from choline-deficient rats. A semipurified diet, with and without choline supplement, and a laboratory ration diet were fed for 11 to 14 days. The fatty acid composition of the renal lipids from these animals was determined by gas-liquid chromatography. The fatty acid patterns of the total lipids from severely hemorrhagic kidneys showed a striking decrease in the relative amounts of linoleic and arachidonic acids when compared with values obtained with control animals. Changes in the fatty acid composition of the renal phospholipids accounted for much of the difference observed in the total lipids. The values obtained for kidneys with less severe lesions were intermediate between the extremes noted in normal and severely affected kidneys. The fatty acid changes in the abnormal kidneys appear to be related to the severity of the syndrome.

The acute renal lesion involving hemorrhagic degeneration which results from a deficiency of choline in the diet of weanling rats was first described by Griffith and Wade (1), and subsequent investigations related to this syndrome have been fully reviewed (2–4). To date no specific unequivocal explanation has been formulated to explain the histological alterations in the renal structure. The most significant manifestations of the deficiency include stainable fat droplets in the epithelial cells of the proximal convoluted tubules of the cortex, tubular ischemia and necrosis, and hemorrhage in the capsule and at the periphery of the cortex (5). Glomerular degeneration always follows the latter as a secondary event (6). The primary stage which precedes tubular necrosis consists of lipid droplet formation associated with swelling of the affected nephrons (7). It is not known whether these renal fatty changes are an effect of deficiency of choline compounds in the affected tubules or a result of changes in other substances less directly related to "one-carbon" metabolism.

The kidney requires a continuing source of either choline or of sources of "one-carbon" units for the synthesis of lecithin. It has been shown that in the pre-lesion condition both the concentration and amount of phospholipid per kidney are markedly reduced below the corresponding levels for choline-fed animals, thereby implying a direct link between a decrease in phospholipid in the kidneys and the onset of renal disease (8, 9). The present exploratory survey was undertaken to obtain additional information related to changes in the lipid composition of hemorrhagic kidneys compared with normal kidneys. Such observations might lead to a better understanding of the formation of fat droplets in the renal lesions during choline deficiency.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain, between 40 to 60 g in weight, were used. During the third week of lactation dams and their litters were fed a vitamin B_{12}-deficient diet which was normal with respect to other nutrients and included 0.28% choline chloride. All the animals used in these experiments were fed this diet. The vitamin B_{12} restriction prior to weaning was imposed to minimize the *de novo* synthesis of choline from "one-carbon" units. Strength et al. (10) had observed the choline-sparing action of this vitamin and their results were confirmed by Best et al. (11) who developed a diet which could be used to study the sparing effect of vitamin B_{12}. The pro-

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tective action of vitamin B_{12} on the subsequent development of choline deficiency has been demonstrated also by a marked decrease in the incidence of hemorrhage in the kidney when the vitamin was added to the mother's diet during the period from birth to weaning (12). In the present studies the pre-weanling animals were not deprived of choline itself because the double deprivation of vitamin B_{12} and choline would have accelerated and intensified the occurrence of renal lesions in those animals subsequently fed the semipurified choline-deficient diet and this would have decreased survival through the planned experimental period.

Materials and diets. The dietary constituents were obtained from a single source [2] with the exception of commercial brands of sucrose, lard, corn oil and peanut oil.

The pre-weanling diet had the following composition per 100 g: (in grams) casein, 20.0; cellulose, 5.0; cornstarch, 42.7; sucrose, 13.0; lard, 13.0; corn oil, 1.60; peanut oil, 0.4, containing fat-soluble vitamins; salt mixture, 4.0; vitamin mixture, 0.027; and choline chloride, 0.28. The salt mixture had the following composition per 100 g of diet: (in grams) $CaCO_3$, 1.18; $CaHPO_4$, 0.54; KH_2PO_4, 1.07; NaCl, 0.46; KCl, 0.29; $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 0.19; $MgSO_4$, 0.14; $FeSO_4 \cdot 7H_2O$, 0.11; $CuSO_4 \cdot 5H_2O$, 0.003; $MnSO_4 \cdot 5H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.003; $CoSO_4 \cdot 7H_2O$, 0.04; NaF, 0.0003; and KI, 0.003. The water-soluble vitamin mixture had the following composition per 100 g of diet: (in grams) thiamine, 0.002; riboflavin, 0.004; pyridoxine, 0.002; niacin, 0.015; and Ca pantothenate, 0.004. The peanut oil in 100 g of diet contained 10 mg α-tocopheryl acetate; 5 mg menadione; 200 units vitamin A; and 28 units vitamin D. The weanling semipurified diet had the following composition per 100 g: (in grams) casein, 8.0; cellulose, 5.0; dextrose, 10.0; sucrose, 56.5; lard, 13.0; corn oil, 1.60; peanut oil (containing fat-soluble vitamins), 0.4; salt mixture, 4.0; vitamin mixture, 0.027; cystine, 0.48; threonine, 0.49; tryptophan, 0.20; phenylalanine, 0.34; and choline chloride, 0.28 (when supplemented at the expense of dextrose for the semipurified control group diet). The salt mixture and the vitamin mixtures had the same composition as the pre-weanling diet. Throughout the experiment all rats were given food and water ad libitum.

The animals in a third group were fed a commercial brand of rat ration [3] containing 0.36% methionine and 0.17% choline chloride to maintain normal kidneys. The fatty acid composition of the post-weanling diets is shown in table 1.

Treatment of kidneys. Individual rats fed the choline-deficient diet were weighed daily from the seventh day after weaning. They were decapitated 11 to 14 days after weaning when the body weight remained steady or showed a decrease on consecutive days. Control animals were killed at the same time as the experimental animals. The kidneys were decapsulated and wiped free from blood. The peripelvic fat was removed from the kidneys before they were weighed. They were covered with methanol in Pyrex tubes, placed in a hot water bath for exactly 2 minutes to destroy enzyme activity and were stored under an atmosphere of nitrogen at −15°.

The solvents used for the storage, extraction and chromatography of tissues and tissue extracts were degassed before use with prepurified nitrogen and all procedures were carried out under an atmosphere of nitrogen to reduce fatty acid oxidation. The kidney lipids from each animal were extracted from the tissue with three successive volumes of approximately 9 ml of chloroform-methanol (2:1) in a ground glass homogenizer. The combined extracts were washed according to the method of Folch et al. (13). The lipids and interface material were com-

TABLE 1

Fatty acid composition of rat diets

Fatty acid	Laboratory ration	Semipurified diet
	%	%
Myristic	3.1	1.9
Palmitic	27.6	25.3
Palmitoleic	4.1	2.6
Stearic	9.0	15.6
Oleic	31.3	40.5
Linoleic	24.9	14.1

[1] Amount of each fatty acid expressed as % of the total fatty acid.

[2] Nutritional Biochemicals Corporation, Cleveland.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

bined, dried with magnesium sulfate and stored in chloroform-methanol (2:1) at −15°.

Suitable aliquots of the total lipid extracts were converted to the corresponding methyl esters of the component fatty acids by acid-catalyzed transesterification. The fatty acids were methylated by heating at 65° for 4 hours in 0.2 ml of 2,2-dimethoxypropane, 0.25 ml of H_2SO_4 and 5 ml of methanol in Teflon-lined screwcapped Pyrex tubes under an atmosphere of nitrogen. The fatty acid analyses were carried out using a Loenco Model 15 B gas chromatograph, with a 183-cm (6-foot) column of 20% diethylene glycol succinate on Chromosorb W. The operating temperature was 197° with a gas flow of 60 ml per minute through a column of 6-mm (¼ inch) diameter.

Total lipid extracts were separated into neutral lipids and phospholipids on chromatographic columns (2 cm I.D.) with 15 g silicic acid containing 10% Celite as the stationary phase according to the method of Fillerup and Mead (14). The eluates were taken to smaller volumes, centrifuged to ensure removal of traces of silicic acid and the dry weights of the lipid solutes were obtained after complete purging from solvents. Precautions were taken to ensure that exposure of the samples to the air was reduced to a minimum. The methyl esters of the fatty acids of the neutral lipids and phospholipids were prepared by the same procedure used for the total lipids. A more sensitive Barber Colman Model 10 gas chromatograph was used for the analysis of the neutral lipids to conserve the limited supply of these materials. This instrument was equipped with a 91-cm (3-foot) column of 13% ethylene glycol succinate on siliconized Chromosorb. The operating temperature was 192° with a gas flow of 70 ml per minute through a column of 6-mm diameter. Fatty acid mixtures analyzed for percentage composition on both gas chromatographs gave identical results.

The linearity of the detector and the weight response for fatty acid methyl esters of different chain length were verified by determining the quantitative composition of mixtures of standards of known composition. For the identification of the fatty acid methyl esters, relative retention times were compared with standards obtained from the Hormel Institute, Austin, Minnesota. The peak area was determined by multiplying the peak height by its width at half-height. The results are expressed as the percentage fatty acid of the total by weight which is a function of the peak area produced by the fatty acid.

RESULTS

Data obtained for total renal lipids are shown in table 2. Although the major effort in the present studies was concerned with a comparison between the lipids of normal and severely hemorrhagic kidneys, a number of animals fed the choline-deficient diet were also obtained during the 11- to 14-day post-weanling period whose kidneys in appearance could be classified as normal, partially recovered or frosted, the categories previously described by Griffith to differentiate the successive stages of degeneration, regeneration and repair of hemorrhagic kidneys (15). The data for these tissues are included for purposes of comparison with the more extensively studied kidneys.

There was a mean increase in kidney weight ranging from 0.94% body weight for the choline-supplemented control animals to 1.84% body weight for the severely hemorrhagic kidneys of the choline-deficient animals with intermediate values corresponding to different stages of degeneration, regeneration and repair. The mean value for the weight of kidneys of the animals fed laboratory ration (0.98% body weight) was essentially the same as that for the kidneys of the control animals.

The total kidney lipids decreased from 4.48 g to as low as 3.10 g/100 g kidney when choline was omitted from the semipurified diet, with intermediate values for the different stages of the syndrome (table 2). Kidneys with normal appearance had less total lipid per 100 g kidney than kidneys from animals fed either the choline-supplemented control or the laboratory ration diet.

The relative amounts of the major fatty acid components found in the kidney total lipids are shown in table 2. A striking difference is observed between the fatty acid composition found in lipids from

TABLE 2

Weight and fatty acid composition of total lipids of kidneys of normal and choline-deficient rats

	Laboratory ration	Semipurified diet with choline	Semipurified diet without choline			
			Appearance of kidneys			
			Normal [1]	Severely hemorrhagic	Partially recovered [1]	"Frosted" [1]
Kidney wt, % body weight	0.98 ± 0.01 [2]	0.94 ± 0.07 [3]	0.96 ± 0.02	1.84 ± 0.08 [2]	1.19 ± 0.05	1.50 ± 0.99
Total lipid/100 g wet kidney, g	4.32 ± 0.19 [4]	4.48 ± 0.24 [3]	3.63 ± 0.51	3.10 ± 0.18 [5]	3.32 ± 0.33	3.10 ± 0.34
Fatty acid [6]						
Palmitic, %	24.1 ± 0.2 [4]	24.1 ± 0.5 [3]	28.8 ± 2.3	33.9 ± 1.0 [5]	26.7 ± 1.0	29.5 ± 0.9
Stearic, %	19.8 ± 0.4	15.0 ± 0.1	19.8 ± 1.6	18.6 ± 0.4	18.5 ± 0.5	18.3 ± 0.9
Oleic, %	14.1 ± 0.2	16.2 ± 0.1	19.3 ± 1.9	25.6 ± 1.0	20.9 ± 0.5	21.4 ± 1.4
Linoleic, %	15.8 ± 0.4	16.2 ± 0.1	13.1 ± 0.5	9.0 ± 0.6	10.9 ± 1.9	8.7 ± 0.7
Arachidonic, %	26.3 ± 0.5	26.7 ± 0.2	25.9 ± 1.2	13.0 ± 1.2	25.9 ± 0.7	22.1 ± 1.3

[1] Mean of 5–9 animals ± SE.
[2] Mean of 36 animals ± SE.
[3] Mean of 17–19 animals ± SE.
[4] Mean of 17 animals ± SE.
[5] Mean of 16 animals ± SE.
[6] Amount of each fatty acid expressed as % of the total fatty acid quantity.

kidneys from control rats and severely hemorrhagic kidneys. The fatty acids from severely hemorrhagic kidneys show an increase in the percentage of palmitic and oleic acids and a decrease in linoleic and arachidonic acids. In the case of arachidonic acid the percentage is about one-half of the control value. The proportion of linoleic acid (9.0%), is likewise appreciably less than that found in the controls (16.2%). Data for kidneys from animals fed the laboratory ration show a renal fatty acid pattern, in particular with respect to linoleic and arachidonic acids, which is essentially the same as that for kidneys of control animals.

Data for kidneys showing less severe stages of the syndrome indicate fatty acid patterns which are intermediate between the extremes noted. The "frosted" group represents the most severely affected kidneys in this series, as is demonstrated by the increased kidney weight (1.50% body weight). The proportion of linoleic acid in the total renal lipids of these animals (8.7%) is significantly lower than in the controls (16.2%). The kidneys with normal appearance have a renal fatty acid pattern with respect to linoleic and arachidonic acids which is essentially the same as in the kidneys of control rats and those fed laboratory ration. The change in the fatty acids noted in the total lipids of severely hemorrhagic kidneys is reflected primarily in the phospholipids (table 3) and to a slight degree in the neutral lipids (table 4) where some decrease is observed in the percentage of linoleic acid but not arachidonic acid.

The data in table 3 illustrate the similarity between the fatty acid patterns of the kidney phospholipids, especially linoleic and arachidonic acids, from animals fed the laboratory ration and control diets. Both groups show significantly greater percentages of these acids than the choline-deficient animals with severely hemorrhagic kidneys. The arachidonic acid values in the phospholipid fractions of the animals fed laboratory ration are somewhat lower than would be expected on the basis of the total lipid fatty acid determination. This is undoubtedly the result of some loss by oxidation even though whenever possible procedures were performed

TABLE 3

Fatty acid composition of phospholipids of kidneys of normal and choline-deficient rats

	Laboratory ration	Semipurified diet with choline	Semipurified diet without choline
Phospholipid/100 g wet kidney, g	3.72 ± 0.29 [1]	3.33 ± 0.39 [2]	2.04 ± 0.45 [3]
Phospholipid/100 g total lipid, g	84.9 ± 1.05 [1]	81.6 ± 1.69 [2]	67.1 ± 1.48 [3]
Fatty acid [4]			
Palmitic, %	26.8 ± 0.5 [1]	19.5 ± 2.0 [2]	35.5 ± 1.2 [3]
Stearic, %	22.5 ± 0.4	17.3 ± 0.6	20.3 ± 0.9
Oleic, %	13.9 ± 0.8	15.3 ± 0.7	20.1 ± 1.2
Linoleic, %	14.5 ± 0.5	16.8 ± 1.2	7.6 ± 0.4
Arachidonic, %	22.3 ± 0.7	28.3 ± 1.4	14.9 ± 1.6

[1] Mean of 15–16 animals ± SE.
[2] Mean of 14–15 animals ± SE.
[3] Mean of 18–19 animals ± SE.
[4] Amount of each fatty acid expressed as % of the total fatty acid quantity.

TABLE 4

Fatty acid composition of neutral lipids of kidneys of normal and choline-deficient rats

	Laboratory ration	Semipurified diet with choline	Semipurified diet without choline
Neutral lipid/100 g wet kidney, g	0.60 ± 0.05 [1]	0.97 ± 0.06 [2]	1.05 ± 0.10 [3]
Neutral lipid/100 g total lipid, g	15.1 ± 0.97 [1]	18.4 ± 1.82 [2]	32.9 ± 1.51 [3]
Fatty acid [4]			
Palmitic, %	38.0 ± 1.9 [1]	32.5 ± 1.7 [2]	36.2 ± 1.1 [3]
Palmitoleic, %	6.1 ± 0.4	5.0 ± 0.5	3.5 ± 0.3
Stearic, %	10.0 ± 1.1	12.4 ± 0.5	7.6 ± 0.4
Oleic, %	22.9 ± 1.2	31.5 ± 1.6	37.3 ± 0.9
Linoleic, %	11.3 ± 1.0	11.1 ± 0.8	7.5 ± 0.3
Arachidonic, %	8.6 ± 1.0	6.3 ± 0.6	7.4 ± 0.9

[1] Mean of 16 animals ± SE.
[2] Mean of 14–19 animals ± SE.
[3] Mean of 18–19 animals ± SE.
[4] Amount of each fatty acid expressed as % of the total fatty acid quantity.

under an inert atmosphere. The fatty acid composition of the phospholipids from normal kidneys of control rats and those fed laboratory ration is in good agreement with data reported by Veerkamp et al. (16). The values for the renal phospholipids and neutral lipids (tables 3, 4) show that the neutral lipids make up 15% and 18.4% by weight of the total renal lipids from animals fed laboratory ration and control diets, respectively. The neutral lipids from severely hemorrhagic kidneys make up 32.9% by weight of the total lipids.

DISCUSSION

In the present study the correlation between increased weight of hemorrhagic kidneys and severity of the syndrome agrees with earlier observations (15). This increase in the size of the hemorrhagic rat kidney has been ascribed by Baxter and Goodman (17) as due largely to an increase in substances other than lipid. During the period prior to the onset of renal necrosis, they observed that there is no increase in the concentration of renal lipids and only a little stainable fat in the form of collections of small droplets as observed in frozen sections of these kidneys. Patterson and McHenry (9) reported similar results concerning the concentration of total renal lipids during the early days of choline deprivation, but they noted a decreased concentration of these lipids in later stages of kidney degeneration. They also demonstrated that the water content of normal and hemorrhagic kidneys is essentially the same (9). Hartroft (7), in a study of the day-by-day de-

velopment of the renal syndrome, showed that the first histological change involved sudanophilic droplets of fat formed intracellularly just within the peripheries of the proximal convoluted tubules. A later stage of renal degeneration is manifested by the appearance of necrotic tubules and the concurrent existence of tubules filled with fatty droplets. In the present study the lower amount of total lipid per unit weight of tissue in the severely hemorrhagic kidneys compared with the kidneys of control animals is in agreement with the quantitative results of Patterson and McHenry (9). These workers also reported a decrease in the phospholipid concentration of hemorrhagic kidneys. A similar observation was made in the present work. The resulting increase in the neutral lipid-to-phospholipid ratio of kidneys with severe lesions may relate to the heavy lipid deposits observed by Hartroft in pre-necrotic tubules (7). The rate of phospholipid turnover in the rat kidney is known to be greatest at the period when the incidence of hemorrhagic kidney is most prevalent (18).

The present studies indicate that the hemorrhagic kidney caused by choline deprivation also involves changes in the fatty acid moieties of the renal lipids. Linoleic and arachidonic acids make up a lower proportion of the total fatty acids found in the lipids of severely hemorrhagic kidneys when compared with values for kidneys from control rats or those fed laboratory ration. This decrease is related primarily to the lower percentage of these polyunsaturated fatty acids in the phospholipids, rather than the neutral lipids. Animals fed the semipurified diet, with or without choline supplementation, showed near normal renal fatty acid patterns when the kidneys did not exhibit the severe form of the syndrome. Thus, the change in renal fatty acid pattern appears to be related to the hemorrhagic condition of the kidney.

The steady-state distribution of acids among the glycerolipids in mammals is controlled by many factors (19), and variables such as the diet may control the steady-state glycerolipid composition of some tissues. Dietary choline and metabolic products derived from it are involved in the cytidinediphosphocholine:diglyceride cholinephosphotransferase system (20) as well as the N-methyltransferase reactions (21, 22). Both of these enzymatic systems are concerned with the biosynthesis of lecithin, a major phospholipid in mammalian tissues. Choline deprivation in rats undoubtedly initiates a complex sequence of biochemical events leading to the hemorrhagic syndrome observed in the kidney. The present studies suggest that the turnover of fatty acids in phospholipids may be involved in this choline-dependent sequence of events.

Fatty acids are not distributed evenly between the 1- and 2-positions of the phosphoglycerides. Lands and Hart (23) and Lands and Merkl (24) have shown that in mammals the acyl-CoA:acylglycerophosphorylcholine acyltransferases preferentially esterify saturated fatty acids at the 1-position and unsaturated fatty acids at the 2-position. Since different enzymes esterify these 2 positions on the glycerol moieties of phospholipids, their respective response to control mechanisms need not be the same. For example, it is believed that the ratio of saturated to unsaturated fatty acids in phospholipids can be influenced in some tissues by the concentration of acyl-CoA derivatives since these substances have a selective inhibitory effect on enzymes responsible for fatty acid turnover in phospholipids (19, 23, 24). The complexity of such regulatory mechanisms concerning variations in the phospholipid species of specific tissues will have to be understood in detail at the enzyme level before the relationship observed here concerning the lipid aberrations in hemorrhagic kidneys is understood.

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Uptakes of Zinc, Manganese, Cadmium and Mercury by Intact Strips of Rat Intestine

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ABSTRACT The concentration of 14 metals in the intestinal walls of normal rats was measured by emission spectrography. In vitro uptakes of divalent Zn, Mn, Cd, and Hg, by intact strips of rat intestine were studied as functions of: region of small intestine, incubation time, medium metal concentration, and mutual competition. The uptakes of D-glucose and L-alanine were depressed by Cd and Hg but not by Zn or Mn. The regional uptakes of Zn, Cd, and Hg were least by the jejunum; Mn uptake was least by the ileum. Jejunum and ileum preparations took up Zn > Hg > Cd > Mn. Zinc and Mn uptakes were biphasic, with initial rapid uptakes followed by slower continued uptakes for one hour; in contrast, Hg and Cd uptakes were initially rapid but remained constant and presumably surface-bound. Striking observations were the enhancement of Zn uptake by Hg or Cd and Hg uptake by Zn or Cd, depression of Mn uptake by Zn or Cd and Cd uptake by Hg. Manganese had no effect on Zn or Cd uptake, but Hg uptake was slightly enhanced by Mn (10^{-4} to 10^{-2} M). Cadmium uptake was enhanced by Zn (10^{-5} to 10^{-3} M) but depressed at higher Zn concentrations.

Soft tissues, especially liver, pancreas, and kidney, accumulate transition and related metals (1). It has been reported that Zn concentrates in fish liver to high levels (2); Mn localizes in mitochondria of liver and kidney (3); Hg and Cd accumulate in the kidney and liver (4–12); and Cd distribution in mice resembles that of Zn (7, 10). However, basic data pertaining to the nature of divalent metal ion uptakes by intestinal tissue are still scanty. The mechanisms by which divalent metals are taken up by cell surfaces, transported across cell membranes and ultimately localized in tissues and organs remain obscure.

With in vitro experiments, we have studied the uptake characteristics of a pair of divalent essential metals, Zn and Mn, and a pair of divalent nonessential metals, Cd and Hg. Data presented describe the basic nature of their uptakes, their regional uptakes, relative rates of uptake, competition with one another for uptake, and their effects on the uptakes of D-glucose and L-alanine. Emission spectrographic analyses were performed to measure the initial metal content of rat intestinal tissue prior to incubation with particular metal ions.

METHODS AND MATERIALS

Spectrographic analysis. Eight normal adult rats of the Wistar strain, of both sexes (200 to 300 g), fed a commercial laboratory ration and tap water [3] ad libitum, were fasted for 24 hours, decapitated, and the small intestines immediately removed. The duodenum, jejunum, and ileum from each rat was analyzed separately. Tissues were washed with physiologic saline, everted, rewashed, and blotted gently with Whatman no. 43 filter paper. The average weights of the duodenal, jejunal, and ileal preparations from each rat were 360, 824, and 724 mg, respectively. The samples were placed into 50-ml silica dishes, oven-dried for 16 hours at 110 to 120°, and ashed for 12 hours at 300° and then for 48 hours at 450 ± 10°.

Ash from each sample was mixed with an equal weight of graphite, using Pd as

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[3] Purina Laboratory Chow analysis: (ppm) Zn, 59.08; Mn, 50.49; Cd, 0.40; Hg, 0.88; Ralston Purina Company, St. Louis. Water analysis (μg/liter): Zn, not detected; Mn, 4.3; Durfor, C. N., and E. Becker 1964 Public water supplies of 100 large cities of U.S. Geol. Survey Water Supply Paper no. 1812, p. 230.

the internal standard. Duplicate aliquots of each were DC anode-excited at 11 A short circuit, using a Jarrell-Ash emission spectrograph (3.4 m Ebert Mark IV), with a diffraction grating of 22,500 lines/2.5 cm, a 76-cm camera and Kodak 103a-O spectroscopic plates. A standard matrix, simulating tissue ash, was prepared with the following: (in %) 18.6 K as KPO_3, 15.0 Na as NaCl, 1.0 Mg as MgO, 1.0 Ca as $CaCO_3$, and 0.98 Fe as Fe_2O_3. A mixture of 15 elements was added to the matrix prior to arcing, and dilutions were made to cover the range of 0.5 to 4000 μg/g matrix. A Jarrell-Ash densitometer was used for microphotometry.

Incubation and counting technique. Normal adult rats of the Wistar strain, of both sexes (200 to 300 g), fed a commercial laboratory ration and tap water [4] ad libitum, were fasted 24 hours before killing. The procedure used for the preparation and incubation of tissue throughout these experiments was that of Crane and Mandelstam (13) as modified by Sahagian (14). The tissue samples consisted of 90 to 100 mg of intact strips of rat small intestine. Samples were incubated in 25-ml Erlenmeyer flasks containing 3 ml of Ca-free Krebs-Ringer phosphate (pH 7.4) or Tris 10 mM buffer (pH 7.4) unless otherwise indicated (15, 16). All incubation media were 5 mM in D-glucose or, in one series of experiments, 5 mM in L-alanine. No more than one of the metals studied was added to any one incubation medium except in experiments testing the effect of one metal on the uptake of another. These metals were in the form of their divalent cations; the symbols used are to be so interpreted. All incubations were carried out at 37°. The gas phase was pure O_2, except for the anaerobic experiments when pure N_2 was used. Unless otherwise indicated, all measurements of uptake were performed on at least 3 aliquots of tissue at each metal concentration, and all results cited are the mean of such triplicate analyses. The variability within triplicate determinations of uptake for the same metal was less than ± 5%; the variability among different experiments conducted under identical conditions was less than ± 8%. The apparent differences in the uptakes for the same metal, expressed in counts per minute, resulted largely from the use of different levels of specific activity. Control samples were carried out simultaneously for each experiment.

Nuclear-Chicago radiation analyzer and scaler was used to measure gamma-emission. For uniformly labeled D-glucose and L-alanine measurements, tissue samples were ground with a Potter-Elvehjem homogenizer and proteins precipitated with 80% ethanol. An aliquot of each supernatant was transferred to a 3-cm diameter planchet, dried under an infrared lamp, and beta-emission was measured with a Nuclear-Chicago scaler. All readings were corrected for background.

Compounds and coventions used. Analytical reagent grade D-glucose, L-alanine, and the chlorides of Zn, Mn, 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 and ^{14}C-L-alanine were obtained from the International Chemical Nuclear Corporation.

Spectrographic results are expressed as micrograms of metal per gram of wet weight of tissue. All other calculations are based on 100 mg wet weight of tissue determined prior to incubation.

RESULTS

Metal content of normal rat intestine. Concentrations of 14 metals in rat duodenum, jejunum, and ileum were determined by emission spectrography (table 1). Metals present appeared to fall into 3 groups of relative abundance. Iron and Zn with median concentrations in excess of 33 μg/g tissue were the most abundant. Copper, Mn, Al, and Mo, had median concentrations of 5.9 μg to 0.73 μg/g tissue. Nickel, Ti, Sn, Pb, Cr, Ba, Sr, and Ag, in the order of decreasing abundance, had median concentrations of 0.19 μg to 0.01 μg/g of tissue. The concentrations of Zn and Mn were different; Zn was 30 times as concentrated as Mn, despite the dietary supply levels of these 2 metals being about the same.[5] Cadmium was detected in only

[4] See footnote 3.
[5] See footnote 3.

TABLE 1

Emission spectrographic analysis of rat intestine for metals from 8 rats analyzed individually for each metal

	Zn	Fe	Cu	Mn	Al	Mo	Ni	Ti
	μg/g wet tissue		*μg/g wet tissue*		*μg/g wet tissue*		*μg/g wet tissue*	
Duodenum								
Mean	55 ± 12 [1]	69 ± 5	5.3 ± 0.7	2.20 ± 0.56	1.81 ± 0.22	0.72 ± 0.14	0.212 ± 0.041	0.180 ± 0.050
Median	50	68	5.9	1.60	2.10	0.90	0.190	0.140
Range [2]	21–73	52–89	2.9–7.5	0.67–4.00	1.20–2.60	0.23–1.10	0.090–0.310	0.081–0.220
Jejunum								
Mean	52 ± 17	42 ± 3	4.5 ± 0.5	1.10 ± 0.12	0.91 ± 0.23	0.75 ± 0.12	0.136 ± 0.026	0.132 ± 0.006
Median	34	43	4.8	1.20	0.87	0.84	0.130	0.130
Range	22–91	34–53	3.1–5.4	0.66–1.50	0.45–1.20	0.24–1.10	0.066–0.170	0.120–0.150
Ileum								
Mean	54 ± 13	42 ± 3	4.8 ± 0.6	1.60 ± 0.26	1.10 ± 0.18	0.61 ± 0.08	0.138 ± 0.025	0.138 ± 0.009
Median	50	41	5.5	1.30	1.10	0.73	0.130	0.140
Range	28–68	32–48	2.7–5.8	0.31–1.64	0.55–1.50	0.29–0.81	0.076–0.200	0.110–0.170

	Sn	Pb	Cr	Ba	Sr	Ag
	μg/g wet tissue		*μg/g wet tissue*		*μg/g wet tissue*	
Duodenum						
Mean	0.190 ± 0.26	0.113 ± 0.24	0.028 ± 0.009	0.091 ± 0.016	0.044 ± 0.010	0.015 ± 0.003
Median	0.180	0.110	0.005	0.080	0.037	0.011
Range	0.110–0.240	0.057–0.160	0.005–0.077	0.031–0.130	0.009–0.100	0.010–0.022
Jejunum						
Mean	0.076 ± 0.007	0.080 ± 0.013	0.079 ± 0.027	0.029 ± 0.009	0.030 ± 0.007	0.014 ± 0.004
Median	0.076	0.071	0.035	0.038	0.034	0.014
Range	0.056–0.081	0.048–0.110	0.005–0.150	0.015–0.059	0.002–0.052	0.008–0.019
Ileum						
Mean	0.091 ± 0.012	0.081 ± 0.017	0.105 ± 0.035	0.036 ± 0.003	0.041 ± 0.006	0.032 ± 0.007
Median	0.110	0.074	0.025	0.039	0.049	0.032
Range	0.049–0.120	0.028–0.130	0.005–0.160	0.028–0.043	0.018–0.062	0.011–0.062

[1] Mean ± SD.
[2] 75% range.

a few samples, indicating that there was usually less than 0.01 μg/g of wet tissue. Mercury was not measured spectrographically because it volatilized during ashing. Variations in the trace metal pattern of the small intestine were apparent; however, no attempt was made to evaluate retention levels of the metals by this tissue under the conditions of these experiments.

Uptake of individual metals by rat intestine. To study regional differences in uptake, tissue from the duodenum, jejunum, and ileum was separately incubated for 30 minutes with 10^{-6} M solutions of each of the metals. Two patterns of uptake were observed: Mn showed little regional variation, whereas, Zn, Cd, and Hg were taken up to a greater extent by the duodenum and ileum than by the jejunum (fig. 1).

To study relative uptakes of these 4 metals, pooled tissue from the jejunum and ileum was incubated for one hour with solutions of each of the metals. Two sets of experiments were conducted, one in which the initial medium concentration of each metal was the same (0.3 μmole/3 ml) and the other in which the initial medium molarity was varied as indicated in table 2. On a comparative basis, metal uptake rates follow the order of Zn > Hg > Cd > Mn by the end of one hour of incubation when the initial concentration of the medium was 10^{-4} M for each respective metal. When initial medium metal concentrations were increased even five- or sixfold as in the case of Zn and Mn, respectively, the tissue metal uptakes increased but the relative order of uptakes still remained about the same.

Uptakes of 10^{-6} M concentrations of the same metals by similar preparations were studied as functions of incubation time (fig. 2). There was an initial rapid uptake of Zn and Mn followed by a slower

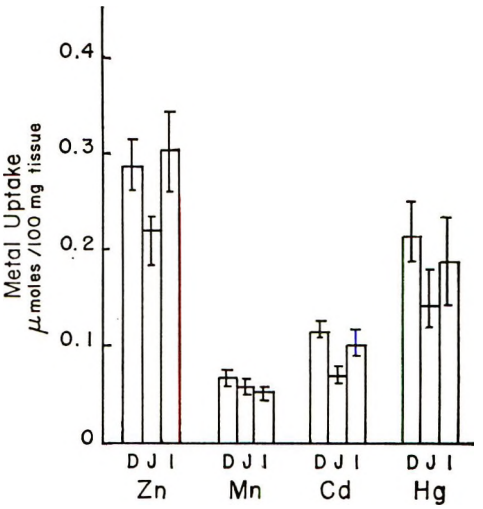

Fig. 1 Uptakes of metal ions by various regions of rat intestine. Intact strips of duodenum (D), jejunum (J), and ileum (I), were separately incubated for 30 minutes with 10^{-6} M labeled Mn, Zn, Cd or Hg. Incubations were made at 37° in Ca-free Krebs-Ringer phosphate buffer (pH 7.4) 5 mM in glucose and under 100% O_2. Tissue samples were blotted after incubation and placed in 15 × 20-mm test tubes containing 1 ml of distilled water and counted. From each of 4 rats, 3 duodenal, 5 jejunal and 6 ileal preparations were made and tested against all 4 metals. Mean and standard deviations of metal uptakes are presented.

TABLE 2

Comparative metal uptakes by rat intestine [1]

	Initial medium conc	Metal uptake	Ratio of initial medium conc	Ratio of metal uptakes
	μmole/3 ml	*μmole/100 mg wet tissue*		
Zn	1.545 0.30	0.446 0.074	5.15	6.03
Hg	0.50 0.30	0.107 0.061	1.67	1.75
Cd	0.975 0.30	0.111 0.052	3.25	2.13
Mn	1.820 0.30	0.120 0.024	6.07	5.0

[1] Pooled intact strips of jejunum and ileum were incubated with metal ions for 1 hour in Krebs-Ringer solution 10 mM in Tris buffer (pH 7.4) and 5 mM in glucose.

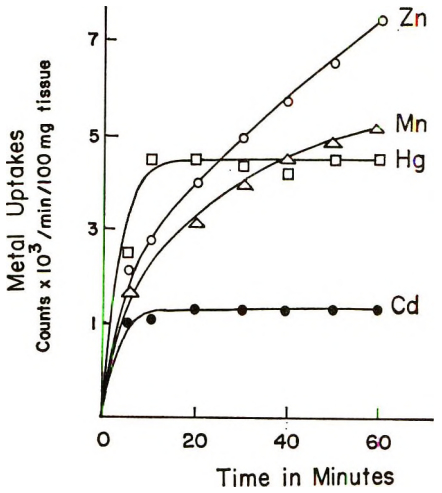

Fig. 2 Aerobic uptakes of metal ions as functions of incubation time. Pooled intact strips of jejunum and ileum were incubated for various periods with 10^{-6} M labeled Mn, Zn, Cd, or Hg. The other conditions of incubation and radiometal assay were as described in the legend to figure 1. Similar curves were obtained under 100% N_2; these are not presented.

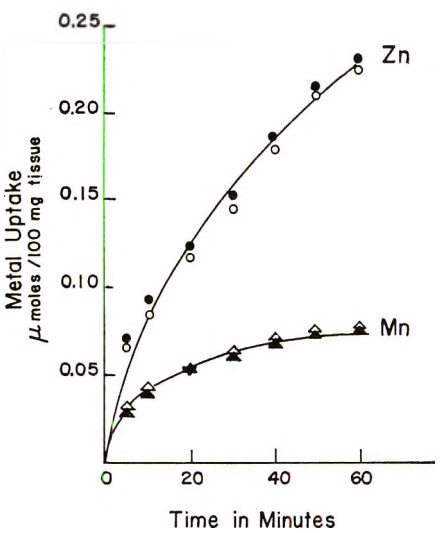

Fig. 3 Uptakes of Mn and Zn in the presence of 2,4-dinitrophenol (10^{-4} M). Pooled intact strips of jejunum and ileum were incubated for 60 minutes in Krebs-Ringer solution 10 mM in Tris buffer (pH 7.4) with 10^{-4} M labeled Mn or Zn. Open figures indicate incubation without, and solid figures incubation with 2,4-dinitrophenol. The other conditions of incubation and radiometal assay were as described in the legend to figure 1.

rate for the remainder of the 60-minute incubation period. The uptake of Cd and Hg began with a similar rapid phase, but after 10 minutes there was no further increase in their uptakes. Uptakes of all 4 metals were unaltered by anaerobic conditions (100% N_2) (fig. 2). Moreover, 2,4-dinitrophenol (10^{-4} M) had no effect on the aerobic uptakes of Zn and Mn (fig. 3).

Uptakes by similar preparations were studied as functions of metal concentration of the medium. For each metal, a tissue concentration was reached which depended on the initial medium concentration of the metal and the characteristic rate of uptake of the particular metal. With initial molarities about 10^{-2}, the uptakes in 2 hours for Hg, Zn, Cd, and Mn were in the approximate ratios of 4:3:2:1. The uptake of each metal responded differently and non-linearly to concentration (fig. 4).

Uptake of one metal in presence of another. The effect of increasing concentrations (10^{-7} to 10^{-2} M) of one metal on the uptake of a second metal (10^{-6} M) was studied. Cadmium or Hg at 10^{-5} to 10^{-3} M greatly enhanced Zn uptake (fig. 5); in contrast, Mn had no similar en-

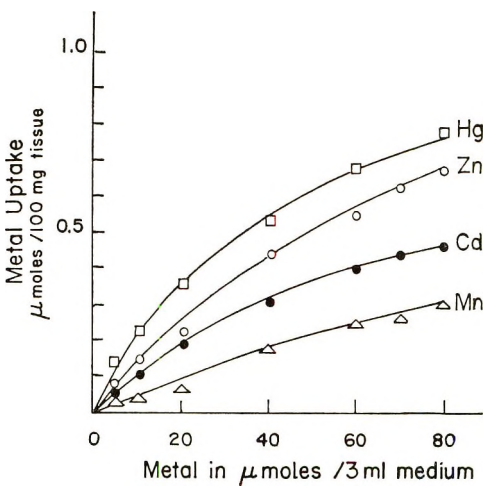

Fig. 4 Uptakes of metal ions as functions of medium metal concentration. Pooled, intact strips of jejunum and ileum were incubated for 2 hours in Krebs-Ringer solution 10 mM in Tris buffer (pH 7.4) with increasing concentrations of labeled Mn, Zn, Cd, or Hg. The other conditions of incubation and radiometal assay were as described in the legend to figure 1.

hancing effect on Zn uptake or Cd uptake (fig. 6) and only slightly enhanced Hg uptake above 10^{-4} M (fig. 7). The uptake of Mn was not altered by Hg, or by Zn up to 10^{-4} M; however, it was depressed by Cd, or by Zn at higher concentrations (fig. 8). Mercury uptake was markedly enhanced by Cd or Zn but little by Mn above 10^{-4} M (fig. 7). Mercury depressed the uptake of Cd, whereas, Zn enhanced Cd uptake up to 10^{-3} M and depressed it above this concentration up to 10^{-2} M (fig. 6).

Effects of metals on D-*glucose and* L-*alanine uptakes.* The effects of metals (10^{-6} to 10^{-3} M) on the aerobic uptake of D-glucose by intact strips of intestine were examined. At these concentrations, Cd and Hg inhibited the aerobic uptake of D-glucose; whereas, Zn and Mn had no similar effect (fig. 9).

The effect of Cd or Hg on the uptake of L-alanine uptake was less than on D-glucose uptake, but inhibition was evident with 10^{-6} M or greater concentrations of Cd or Hg; the latter was more inhibitory. Zinc and Mn had no inhibitory effect (fig. 10).

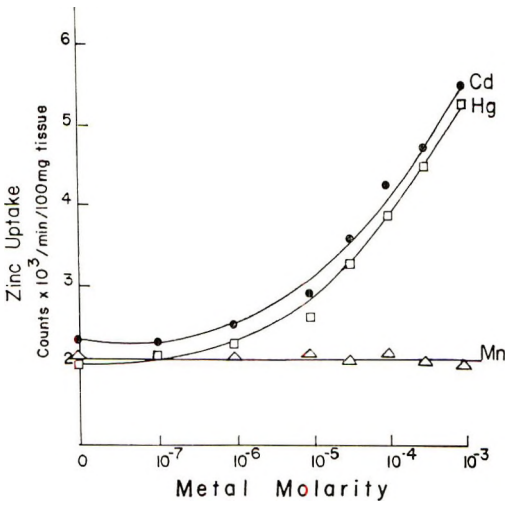

Fig. 5 Effects of Mn, Cd, or Hg on aerobic uptake of Zn. Pooled intact strips of jejunum and ileum were incubated for 30 minutes in Krebs-Ringer solution 10 mM in Tris buffer (pH 7.4) with 10^{-6} M labeled Zn and various concentrations of Mn, Cd, or Hg. The other conditions of incubation and radiometal assay were as described in the legend to figure 1.

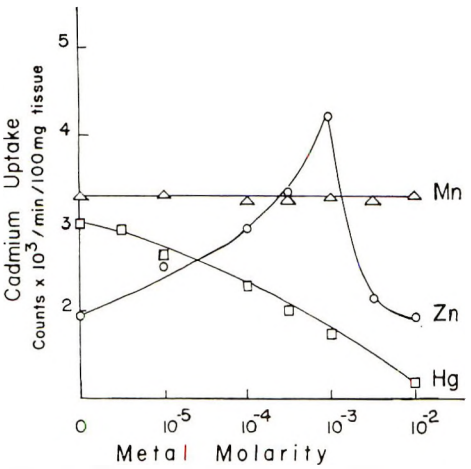

Fig. 6 Effects of Zn, Mn, and Hg on Cd uptake. Pooled intact strips of jejunum and ileum were incubated for 30 minutes with 10^{-6} M labeled Cd and increasing concentrations of the other metals. The other conditions of incubation and radiometal assay were as described in the legend to figure 5.

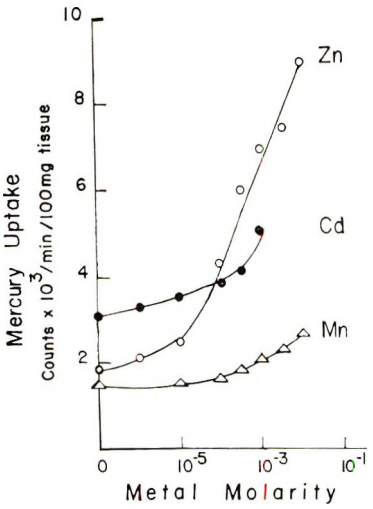

Fig. 7 Effects of Zn, Mn, and Cd on Hg uptake. Pooled intact strips of jejunum and ileum were incubated for 30 minutes with 10^{-6} M labeled Hg and increasing concentrations of the other metals. The other conditions of incubation and radiometal assay were as described in the legend to figure 5.

DISCUSSION

As a first step in our uptake experiments, the concentration of metals present in the walls of the normal rat intestine was measured by emission spectrography to determine the metal content

of this tissue prior to its use in our subsequent metal-uptake experiments. Relatively large amounts of Fe, Zn, and Cu were present. These amounts may well reflect the dietary intake of these metals. No measurements of metal retention based on extended periods of starvation or metal-restricted diets were attempted. Nonetheless, the presence of a large number of metals in intestinal tissue of rats maintained with a normal diet of laboratory ration and tap water was evident. Hence, the possibility that the metals already present may serve to influence the uptake and transport of other metals cannot be ignored. Under the conditions of these experiments, with the exception of Fe, rat intestine contained Zn in greater amounts than the other metals present. There was relatively little Mn in this tissue. The distribution ratio of Zn:Mn (30:1) is in accord with the absorptive characteristics of Zn and Mn observed in our time and concentration experiments and with those reported in marine organisms (2).

In these experiments, only metal uptake was considered. Future studies will take up transport across the intestinal wall. The inhibitory action of Cu and Hg on sugar uptake by intact rat diaphragm and muscle cells was reported by Demis and Rothstein (12). They observed Hg to be more inhibitory than Cu. Our uptake ex-

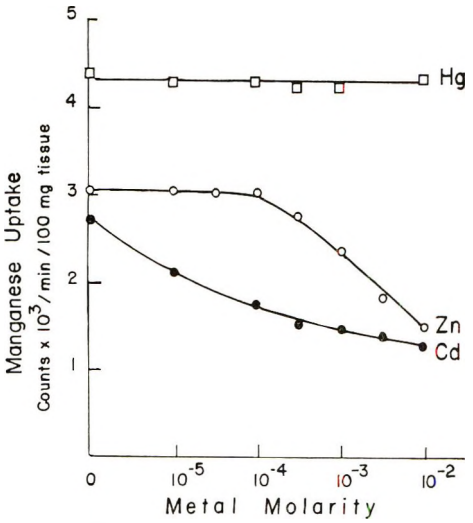

Fig. 8 Effect of Zn, Cd, or Hg on aerobic uptake of Mn. Pooled intact strips of jejunum and ileum were incubated for 30 minutes with 10^{-6} M labeled Mn and increasing concentrations of Zn, Cd, or Hg. The other conditions of incubation and radiometal assay were as described in the legend to figure 5.

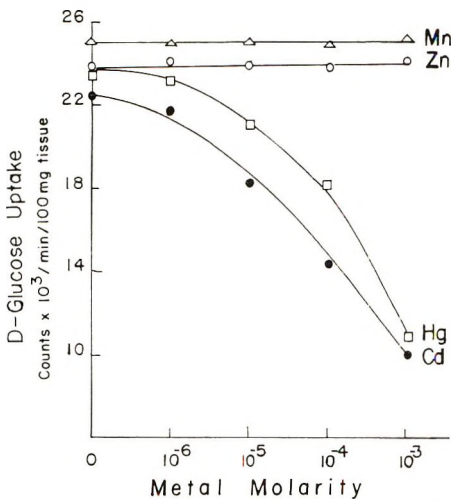

Fig. 9 Effect of metals on aerobic uptake of D-glucose. Pooled intact strips of jejunum and ileum were incubated for 60 minutes with 5 mM uniformly labeled ^{14}C-D-glucose and various concentrations of Mn, Zn, Cd, or Hg. The other conditions of incubation were as described in the legend to figure 1. After incubation, the radiocarbon content of the tissues was measured as described under Methods and Materials.

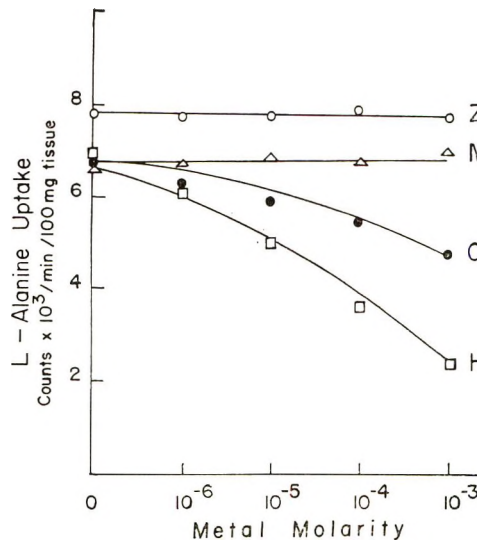

Fig. 10 Effects of metals on aerobic uptake of L-alanine. Conditions were the same as those described in the legend to figure 9, except that 5 mM uniformly labeled ^{14}C-L-alanine was substituted for D-glucose.

periments with D-glucose and L-alanine indicated that only Cd and Hg inhibit the aerobic uptake of these compounds. Thus, Cd showed a closer physiologic relationship to Hg than to Zn; Zinc behaved more like Mn. The inhibition of D-glucose uptake by equimolar concentrations of (10^{-3} M) Hg or Cd was about the same (55%), but inhibition of L-alanine uptake by Hg (59%) was more than twice that by Cd (23%). These results were not entirely unexpected; the uncoupling of oxidative phosphorylation by Cd has been shown by Jacobs et al. (17), and inhibition of sugar absorption by heavy metal cations bound to cell surfaces has been reported by Kamen and Spiegelman (18).

Van Campen and Mitchell (19) reported that with ligated segments, Zn and Fe absorption in rats was more rapid at the duodenum. We observed the jejunum to be the least absorptive section of the rat intestine for Zn, Cd, and Hg. The differences in absorption by the duodenum and by the ileum were not too great. Zinc absorption was somewhat more rapid at the ileum. The duodenal value for Zn uptake was about 14.8% less and the jejunal value about 27.2% less than the ileal value. Manganese was absorbed maximally at the duodenum but at a much slower rate.

Crampton et al. (20) reported that Cu was absorbed maximally in the lower small intestine and that anoxia and 2,4-dinitrophenol (DNP) had no effect on wall uptake of Cu. They concluded that uptake of Cu from the mucosal side resulted from the presence of binding sites on cell surfaces or within cells. Our results with Zn, Mn, Cd, and Hg were similar. The binding affinities of the various metal ions were different (figs. 1 and 4), and their uptakes were not responsive to anoxia or DNP (fig. 3). The very rapid and concentration-dependent uptakes of Cd and Hg coupled with their mutual competition therefore favor the presence of common binding sites on mucosal cell surfaces. Zinc and Mn uptakes were likewise concentration-dependent but also time-dependent. After 2 hours of incubation, when the initial concentrations were the same, 100 mg of tissue contained a greater amount of Hg > Zn > Cd > Mn (fig. 4). Apparently, the total uptake capacity of gut tissue is not the same for all the metals tested (table 2). Gunn et al. (21) have reported that the capacity of the rat testis and dorsolateral prostate for ^{65}Zn uptake is depressed by cadmium. Van Campen (22) has shown that Zn and Cd depressed Cu uptake in rats markedly, whereas Ag and Hg did not produce similar significant depressions. Our experiments have shown marked mutual enhancement and depression between Cd and Hg uptakes. This competitive action took place nearly at all concentrations (10^{-6} to 10^{-2} M). Kägi and Vallee (23) have reported that Zn and Cd compete with each other for a site on metallothionein. The curves obtained (fig. 5) with Cd and Hg clearly indicate their enhancing action on Zn uptake. By contrast, Mn has no similar action on Zn uptake or Cd uptake although Zn and Cd depress Mn uptake (fig. 8). Zinc enhances Cd uptake (10^{-5} to 10^{-3} M).

At the end of various incubation periods, everted intestinal strips were blotted with filter paper, not washed, to remove adhering solution before radioactivity readings were taken. It was noted that some of the mucosal cells with metal ions attached were sloughed off and released into the incubation medium. Under these conditions, washing of this fragile tissue prior to measurement of radioactivity was impractical. Hence, a distinction between true absorption of metal ions and apparent absorption resulting from nonspecific adsorption remains difficult to ascertain. In these experiments, we have studied total metal uptakes by gut tissue and some of the uptake properties of these divalent cations. We have not attempted to differentiate between extracellular adsorption or intracellular accumulation, although the uptakes of Hg and Cd are suggestive that these metals probably act at the cell membrane surface level. Zinc and Mn with time lag in their uptakes and lack of interference with glucose and alanine uptakes follow more closely the pattern of metal activities taking place intracellularly.

Some questions relating to the interactions of these metals with one another and with cell membrane surfaces neces-

sarily arise which are difficult to answer. The depression of Mn uptake by Cd and not by Hg (fig. 8) and the stimulation of Hg uptake by Zn, Cd, and Mn in the same order of magnitude as the individual uptake of these metals are examples (fig. 7). It is also difficult to assess the biological significance of the intestinal uptake properties of the cations studied. But we can point out here that uptake properties of the metals vary considerably so that they can become effective controlling factors in the uptake and transport of essential metals and other substances by the small intestine.

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The Conservation of Water by Young Adult Rats with Restricted Water Supplies [1,2]

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ABSTRACT The purpose of this work was to study the water balance of laboratory rats under restricted water conditions, with particular attention to insensible water loss. Young adult female rats were fed liquid diets by gavage in amounts which maintained constant body weights. The diets were identical except for their water content which was 61.5, 48.3 and 38.7% of the diets. CO_2 production, O_2 consumption and urine osmotic pressure determinations were made in addition to complete water balance studies. The rats adapted to these reduced water intakes without apparent difficulty and reduction of water to 28.1% of the diet during another short trial was also tolerated. A mean urine osmotic pressure of 3855 milliosmoles/liter was found when the diet contained 28.1% water. When the diet contained 38.7% water, the mean urine osmotic pressure was 3581 millisomoles/liter in the main trial and rechecked at 3276 milliosomoles/liter. The greatest saving of water, over the range of water restriction, was made by reduction of urine volume. Decreased dietary water was associated with a decreased proportion of water in the feces. The insensible water loss was less in grams, grams per liter of O_2 uptake or grams per liter of CO_2 production when water was restricted.

Water occupies a special position among the nutrients in several respects. One of these is that the requirements are less precisely defined than for other nutrients. The great variety of factors which influence the requirements for water are in part responsible for this situation. One of these factors which needs further study is the ability of animals to reduce water losses. A reduction in water loss should be equivalent to a reduction in water requirement.

The water-conserving ability of desert-dwelling rodents has been studied extensively, whereas relatively few studies have been made with laboratory rats. Schmidt-Nielsen and Schmidt-Nielsen (1) reported the insensible water loss of certain desert rodents to be distinctly less than it was for laboratory rats or mice. Kirmiz (2) also observed a lesser insensible water loss in kangaroo rats than in laboratory rats when the environmental humidity was high. His work also showed that kangaroo rats had less insensible water loss when fed a dry diet than when moist food was given. Schmidt-Nielsen (3) concluded that the capacity to exhale air at reduced temperature is a mechanism whereby the kangaroo rat avoids water loss. He states that the laboratory rat apparently also has this capability and the smaller water loss of the kangaroo rat may be due to less cutaneous evaporative loss.

A question remains as to whether laboratory rats, a non-desert species, possess the capacity to reduce insensible water loss as a means of water conservation. The work reported here was designed to answer this question and at the same time to make a complete study of the water balance of laboratory rats under conditions of restricted water supply

EXPERIMENTAL METHODS

The animals used in this work were from a strain maintained at this laboratory. All were young adult females about 6 months of age. Body weights ranged from 245 to 360 g.

The main part of the work consisted of a trial using an extra period Latin square design as proposed by Lucas (4). Twelve rats were used, 6 in each of 2 blocks (replications). The treatments consisted of 3 degrees of water restriction and were

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accomplished by giving all water and food combined in liquid diets which were administered by gavage. Each rat was subjected to each treatment for a period of 4 days. The water was restricted in relation to food dry matter, and the diet intake was regulated to maintain body weight as closely as possible. The levels of water restriction used were 1.6, 0.9 and 0.6 parts water to 1 part food dry matter. These rats had been found to take an average of 1.6 g of water per g of food dry matter when allowed the same diet in granular form and water ad libitum. Samples of the liquid diets were taken at each feeding and 4-day composites were made for water determinations. The 3 diets were found to contain 61.5, 48.3 and 38.7% water.

An additional trial was conducted with 7 rats over a 4-day period wherein 2 rats were fed the diet containing 61.5% water and 5 rats were fed a diet made to have a 0.4:1 water:dry matter ratio. This diet was found to contain 28.1% water.

The composition of the basal diet is shown in table 1. All liquid diets were prepared from this basal diet by the addition of the appropriate amounts of distilled water. The liquid diets were stored in tightly capped bottles in the refrigerator and warmed before each feeding. All rats were fed 5 times daily at 2:00 and 6:00 AM, 12:00 noon and 6:00 and 10:00 PM. The solid materials in the diets tended to settle out on standing, and therefore the bottles were thoroughly shaken before the portion for each rat was taken and administered from a 10-ml syringe with a short length of small diameter plastic tubing. The syringe was weighed before and after each portion was fed. The rats had been fed the basal diet for more than a month preceding the start of the trial and had been fed the diet containing 61.5% water on the experimental time schedule during the week preceding the start of the experiment. The amounts of diet required to maintain body weight were determined during this conditioning period so that the body weights were maintained constant with only small adjustments of feed intake during the trials.

TABLE 1

Composition of basal diet

	%
Casein	10
Sucrose	81
Corn oil	5
Salt mixture [1]	4
Vitamin mixture [2]	+

[1] Percentage composition: KH_2PO_4, 37.80; $CaCO_3$, 36.00; NaCl, 13.10; $MgSO_4 \cdot 7H_2O$, 11.00; $FeSO_4 \cdot 7H_2O$, 1.50; $MnSO_4 \cdot H_2O$, 0.40; $ZnCO_3$, 0.08; KI, 0.05; $CuSO_4$, 0.05; and $CoCl_2 \cdot 6H_2O$, 0.02.

[2] Supplied per kg of diet: 2000 IU vitamin A, 3000 IU vitamin D; and (in milligrams) α-tocopherol, 60; menadione, 0.1; thiamine·HCl, 1.3; pyridoxine, 1.2; niacin, 15; Ca pantothenate, 8; riboflavin, 4; folic acid, 2; biotin, 2.5; and (in micrograms) vitamin B_{12}, 50.

Measurements of water and CO_2 output were made using a series of open circuit type animal chambers. The chambers consisted of small-neck plastic bottles approximately 14 cm in diameter and 28 cm in height, with the bottoms cut off. The bottles were inverted, wire screens inserted for floors and the bottoms (tops) fitted with removable, tightly fitting sheets of Plexiglas with air inlet and outlet fitting. Each chamber was equipped with a urine-fecal separator in which both urine and feces were collected under oil. Dry, CO_2-free air, heated to 28° was pulled by suction through the chambers. The air flow rates were adjusted with manometers at the inlet of each cage so that the flow was sufficient to exchange the air within the chamber completely in approximately 12 minutes. The air outlet of each chamber was connected to a series of three 3.2 by 50-cm U-tubes. The first and third tubes contained $CaCl_2$ and the second tube contained a commercial CO_2 absorbant.[4] The rats were maintained continuously in these chambers except for very short intervals when being fed or weighed. The water and CO_2 collecting tubes were by-passed during the time the chambers were open and for sufficient time subsequently for equilibrium to be re-established. Accurate accounts were taken of the time the chambers were open and on by-pass operation. Seven chambers were used, six containing rats and one as a blank for detection of possible water or CO_2 in the incoming air.

The feces were dried to constant weight at 90° to determine fecal water. Samples of urine were also dried to constant weight and the mean values of 95.1, 88.3 and 85.4% water for the samples from rats

[4] Baralyme, McGraw Edison Company, Chicago.

fed the diets with 61.5, 48.3 and 38.7% water, respectively, were used with urine weights to calculate urinary water.

Insensible water loss was determined by the gain in weight of the first $CaCl_2$ filled drying tube in the air train following each chamber.

CO_2 production was determined by the gain in weight of the second and third U-tubes following the chambers, which contained CO_2 absorbent and $CaCl_2$, respectively. The insensible water loss and the CO_2 production were corrected for the length of time the chambers were open each day to give 24-hour values.

The insensible body weight loss was calculated as the body weight change plus the weight of water and food taken in, minus the weight of urine and feces.

The O_2 consumption was calculated as the insensible water plus the CO_2 produced minus the insensible body weight loss.

To get complete figures for water balance, the amounts of metabolic water were calculated as 0.57 times the digestible dry matter of the diet. The constant 0.57 was derived from the diet composition, using the average values for metabolic water of the nutrient classes, assuming complete oxidation.

The osmotic pressure of urine was determined by freezing-point depression using a Fiske osmometer. Values for some samples exceeded the range of the instrument and hence all samples with concentrations greater that 2000 milliosmoles per liter were diluted 1 to 1 with deionized water and the determined pressure was multiplied by two. This was found to cause no error when tested by checking diluted against undiluted samples.

The osmotic pressure of urine was also obtained from an additional 12 rats subjected to these treatments and experimental design, but using metabolism cages rather than the air-tight animal chambers.

Carcass compositions were determined at the termination of the trials. Water was estimated as loss upon drying to a constant weight at 90°. The dry carcass remaining was then crushed, wrapped in cloth and ether-extracted in a large Soxhlet apparatus. The water content was calculated as a percentage of the fat-free (ether-extracted) carcass.

The statistical analyses with the extra period Latin square design produce estimated mean values which are the sum of the overall mean plus the treatment effects. No block means are reported because there were no significant block × treatment interactions. Residual effects were significant in 2 instances. In these cases the means were adjusted to remove residual effects and these are noted in the tables. The significance among means as indicated in the tables was determined by Duncan's (5) multiple range test.

RESULTS AND DISCUSSION

The average water intakes and losses with the 3 treatments are shown in table 2. The rats were able to adjust to water restrictions of these magnitudes without apparent difficulty. No evidence of discomfort or ill health of any kind was observed in any of the rats.

The mean amounts of fecal water were similar on all treatments and were of minor importance in the water economy of these rats. The mean proportions of water in

TABLE 2

Water intake and output of rats at different levels of water restriction

Dietary water, %	61.5	48.3	38.7
Water in food, g/day [1]	16.5	9.7	7.2
Metabolic water, g/day [1]	5.8	5.7	6.3
Fecal water, g/day [1]	0.4	0.3	0.3
Urinary water, g/day [2]	10.1	4.6	3.8 ± 0.32 [3]
Insensible water loss, g/day [2]	11.3	9.8	8.4 ± 0.12

[1] Treatment means including extra period values.
[2] Means not underscored by the same double line differ significantly ($P < 0.01$).
[3] SE.

the feces of the rats receiving the 3 levels of water intake as noted in table 3 showed highly significant differences. Thus rats are capable of conserving water by reduction of fecal losses. Under conditions where the ration is not so highly digestible, this mechanism should provide significant savings of water.

The mean values for urinary water loss shown in table 2 illustrate that the reduction in this avenue of water loss afforded an important water saving to the rats. The similarity of urinary water loss with the 48.3 and 38.7% water diets is at least partly a reflection of the fact that the difference in total water available with these diets is not great. It is noted that although urinary water was not the greatest water loss to the rats with most water available, it was the route of water loss with the greatest decrement when water was restricted. The mean osmotic pressure of urine increased markedly and highly significantly at each level of water restriction. It appears that the urine of rats was concentrated to a considerably greater degree than is usually accomplished with ad libitum water intake. The urine osmotic concentrations observed with the most severely restricted water are in excess of maximal values reported elsewhere for laboratory rats. Radford (6) reported a maximal urine concentration of 2670 milliosmoles/liter by rats with no water but with food available and a mean concentration of 2719 milliosmoles/liter when neither water nor food was available. Bauman et al. (7) observed that rats given access to food but without water produced urine with a mean osmolality of 2984 milliosmoles/kg. A number of desert rodents showed urinary concentrations of greater than 3000 milliosmoles/liter (8). This ability, however, is frequently associated with anatomical features of the kidney not found in laboratory rats (9).

These extremely high values for osmotic pressure of the urine gave rise to the question of whether extensive breakdown of urinary organic constituents might have occurred during a short period of storage in the refrigerator. To explore this possibility, the treatments were repeated with 12 rats in metabolism cages. The urine was collected under toluene and the pressures were determined on the day of collection. The mean urinary outputs and osmotic pressures were as shown in table 4. The differences in urinary output and osmotic pressure with treatments were highly significant. The osmotic pressures were apparently somewhat lower than those observed in the main experiments but the relationship among treatments was approximately the same and the mean osmotic pressure of the urine from the rats fed the diet containing 38.7% water was again greater than previously reported val-

TABLE 3

Water concentrations in feces and urine of rats with different levels of water restriction

Dietary water, %	61.5	48.3	38.7	
H_2O in feces, % [1,2]	57.7	47.5	42.6 ±	1.59 [3]
Urine osmotic pressure, milliosmoles/liter [1]	1664	2851	3581	±136

[1] Means not underscored by same double line differ significantly ($P < 0.01$).
[2] Mean of $47.5 > 42.6$ ($P < 0.05$).
[3] SE.

TABLE 4

Urinary output and osmotic pressure with different degrees of water restriction as determined in metabolism cages

Dietary water, %	61.5	48.3	38.7	
Urine, g/day [1,2]	7.0	4.3	3.2 ±	0.336 [3]
Urine osmotic pressure, milliosmoles/liter [1,4]	1099	2331	3276	±138

[1] Means not underscored by same double line differ significantly ($P < 0.01$).
[2] Mean of $4.3 > 3.2$ ($P < 0.05$).
[3] SE.
[4] Residual effects removed.

ues for laboratory rats. The forcing of food intake with reduced water intake and possibly the combining of food and water intake at one time are the factors which must have caused the increased concentration of urine. The mechanisms by which the increased concentration was accomplished are not known. The work of Ames and Van Dyke (10) indicates that the more concentrated urine of kangaroo rats is produced with a higher concentration of antidiuretic hormone than is observed in laboratory rats. However, Radford (6) did not find that laboratory rats produced urine as concentrated as the urine obtained in this study when he administered exogenous pitressin.

The treatment means for insensible water losses expressed in grams per day are shown in table 2. The reductions of this water loss which occurred with water restriction illustrate that mechanisms to accomplish this water saving are present in non-desert animals not adapted to high temperature or low water environments.

Since insensible water loss represents a heat loss to the animal, a reduction of this loss must be accompanied either by a reduction in heat production or by an increase in heat loss by means other than water evaporation. The data obtained with these rats are not adeqaute to show which of these alternatives was operative. Heat production can be estimated from the O_2 uptake. O_2 uptake, CO_2 production and respiratory quotient values are shown in table 5. Despite the lack of precision of the procedure used for the calculation of O_2 consumption, a small but significant reduction with decreased water intake is shown. Heat production, estimated from the mean O_2 uptake and respiratory quotient values, indicates a reduction at the lowest water intake similar in magnitude to the reduction calculated from the difference in mean insensible water loss multiplied by the heat of vaporization. If heat production were reduced, a reduction in food intake would be expected, provided body weight and composition remained constant. Food intake appears to have been greatest with the lowest water intake as shown by the figures for metabolic water (calculated from dry matter intake) in table 2. Only very small changes in body weight occurred and statistical analysis of these changes did not show any differences with treatments.

Insensible water loss has been related to O_2 consumption by Schmidt-Nielsen and Schmidt-Nielsen (1) and it is assumed that a relatively constant relationship exists. However, Hudson (8) points out that this relationship changes with the environmental temperature. The treatment means for insensible water loss related to both O_2 uptake and CO_2 production are presented in table 6. When water was restricted these rats did not maintain a constant relationship between evaporative water loss and either O_2 uptake or CO_2 production, even though the environmental temperature was constant.

The absence of symptoms of stress or disease in the rats when water was most severely restricted led to the trial wherein 5 rats were given the ration containing only 28.1% water. The mean values for

TABLE 5

O_2 uptake, CO_2 production and respiratory quotient of rats as affected by water restriction

Dietary H_2O, %	61.5	48.3	38.7
O_2 uptake, liters/day [1,2]	7.68	7.63	7.28 ± 0.150 [3]
CO_2 production, liters/day [1,4,5]	7.08	7.35	7.64 ± 0.121
Respiratory quotient [6]	0.94	0.97	1.03 ± 0.017

[1] Significant effect of treatment, $P < 0.05$.
[2] The statistical test for differences among means revealed no significant difference.
[3] SE.
[4] Residual effects removed.
[5] Means not underscored by the same single line differ significantly ($P < 0.05$).
[6] Means not underscored by the same double line differ significantly ($P < 0.01$); $0.97 < 1.03$ ($P < 0.05$).

TABLE 6

Insensible water loss as affected by water restriction

Dietary water, %	61.5	48.3	38.7
Insensible water loss			
g/liter O_2[1]	1.49	1.29	1.16 ± 0.021 [2]
g/liter CO_2[1]	1.58	1.33	1.13 ± 0.023

[1] Means within line differ significantly ($P < 0.01$).
[2] SE.

TABLE 7

Water intake and output and osmotic pressure of urine of rats fed 0.4 g water/g of dry matter [1]

Water with diet	Metabolic water	Urinary water	Fecal water	Insensible water	Osmotic pressure of urine
g/day	*g/day*	*g/day*	*g/day*	*g/day*	*millios-moles/liter*
5.5	7.5	2.9	0.3	7.8	3855

[1] Mean from 5 rats with 4 daily observations on each.

water intake and output as well as urinary osmotic pressure are shown in table 7. The water balances of the 2 rats fed the 61.5% water diet in this trial were similar to the balances obtained with this diet in the main experiment. The rats given the 28.1% water diet showed no signs of distress or disturbance to health even at this low level of water intake. The osmotic pressure of the urine was extremely high, indicating that the kidney of the laboratory rat has an even greater urine concentrating power than was shown in the previous experiment.

The water intake and output figures indicated that the rats remained in water balance even when water was most severely restricted. Maintenance of water balance was further demonstrated by the water content of the rat carcasses at the termination of the trials. Carcass water, as a percentage of the fat-free body, was found to average 72.1, 71.8, 71.1 and 71.5 for the rats terminating with the 61.5, 48.3, 38.7 and 28.1% water diets, respectively. Statistical analysis did not reveal any differences among these means for carcass water.

The restriction of drinking water to laboratory rats leads to a reduction in the food intake and weight gain of growing rats as shown by Crampton and Lloyd (11). Lepkovsky et al. (12) observed that restriction of water reduced the food intake of mature rats with the exception of a group of fat male rats. Some limited tests in this laboratory showed that restriction of ad libitum water supply led to reduced food intake and body weight loss by mature female rats. In contrast with the results with rats, Konishi and McCay (13) noted that the restriction of water did not reduce food intake of adult dogs. Their maximal restriction, however, appears to have been at approximately 1 to 1 water to food dry matter. Lindeborg (14) restricted the water intake of several species of mice and observed that all species lost weight. In the experiments reported here when the water was restricted, with no opportunity given to reduce food intake, the rats maintained body weight and health and remained in water balance. The drinking water intake was restricted to as little as 20% of their ad libitum intakes for some rats. The mean total available water for the rats given the diet with 28.1% water was approximately one-half of what would be expected with ad libitum water intake. The minimal water requirement of laboratory rats for maintenance therefore appears to be considerably less than their ad libitum intake.

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Dietary Carbohydrates and Possible Pre-lesion Biochemical Changes in the Aortas of Adult Male Rats [1]

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ABSTRACT Aortic homogenates from adult male rats were explored by biochemical methods for pre-lesion changes in association with high carbohydrate diets and with starvation-refeeding. Supporting tests included assays for certain blood lipids and for liver glucose 6-phosphate dehydrogenase activity. The liver data indicated overinduction of this enzyme by the high carbohydrate diets; the ratio of free cholesterol to phospholipids in the blood increased during starvation and especially with a high glucose diet, and both of these treatments appeared to produce aortic changes. Reports of similar studies have not been found, and some difficulties of interpretation are discussed. It is suggested that this type of approach may prove fruitful in the search for evidence concerning nutritional contributions to the etiology of cardiovascular disease.

A more direct association of human cardiovascular disease with dietary carbohydrates than with fats has been suggested on statistical (1) and human experimental (2) grounds. Starvation-refeeding has been reported to accelerate the onset of the disease in man (3) and has been made to do so in pigs (4) and rats (5), although not reliably. These animals were refed high glucose diets. The development of frank arterial lesions may not be a primary effect of diet, in that lesions must be preceded by biochemical changes and much of their histopathology may denote secondary changes. We have, therefore, started to investigate biochemical changes in the aortic wall in time-association with diet and with appropriate liver and blood changes, selecting first some aortic parameters in which change has been reportedly associated with lesions or hypertension. These included a reduction of glucose 6-phosphate dehydrogenase (G-6-phcsphate dehydrogenase) activity (6) and increases of alkaline phosphatase (7) and of lactate [3]; that of lactate was reportedly associated with experimental hypertension, and that of alkaline phosphatase with hypermicrovascularization of the vessel wall.

The results of our first exploration, designed to suggest areas for more exact study, appear significant enough to justify a preliminary report.

MATERIALS AND METHODS

Forty-eight male rats of the Sprague-Dawley strain, which on receipt weighed 366 ± 23 g, were caged individually, arbitrarily divided into 3 groups of 16 and fed ad libitum for 2 weeks of which the last day was designated day zero; then 12 rats from each group were starved for 8 days (from day zero), four being killed on day 7. Eight were refed ad libitum, starting on day 8, and were decapitated, four 6 hours and four 72 hours after first refeeding. The 4 rats not starved were killed on day 10. Water was continuously available. Composition of the diets and the feeding schedule are shown in table 1.

Blood was collected in heparinized tubes and the plasma was stored at − 15° and assayed for free cholesterol (8) and total phospholipids (9, 10) which were expressed according to the principle in the

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[3] St. Clair, R. W., N. H. Booth and M. L. Hopwood 1965 Metabolic characteristics of normal and hypertensive swine arteries. Federation Proc., 24 (part 1): 192 (abstract).

TABLE 1

Composition of diets

	High glucose diet (HG)	High starch diet (S)	High fat diet (HF)
	% by weight	*% by weight*	*% by weight*
Casein	20.00	19.50	31.92
Methionine	0.50	0.50	0.82
Glucose [1]	69.71	—	—
Starch	—	70.21	—
Corn oil	5.0	5.0	—
Vegetable fat [2]	—	—	59.42
Salt mix [3]	4.0	4.0	6.55
Vitamin premix [3]	0.50	0.50	0.82
Vitamin E succinate [3]	0.10	0.10	0.16
Vitamins A and D [4]	0.09	0.09	0.15
Choline chloride	0.10	0.10	0.16
Caloric values, kcal/100 g	403.6	403.6	668.7

Feeding schedule

Group (rat nos.)	Diets for 2 weeks before day 0 and of 4 rats/group during days 0 to 10	Refed diets
1–16	High glucose (HG)	high glucose (HG)
17–32	Stock diet and sucrose [5] (CS)	high starch (S)
33–48	Stock diet [5] (C)	high fat (HF)

[1] Cerelose, Corn Products Company, New York, N. Y.
[2] Crisco, Procter and Gamble, Inc., Cincinnati.
[3] As described previously (18).
[4] 1500 IU of vitamin A and 100 IU of vitamin D/g of HG and S diets; 2500 and 167 IU, respectively, of vitamins A and D/g of HF diet.
[5] A proprietary stock diet (Teklad, Inc., Monmouth, Ill.) guaranteed to contain 24% crude protein, 4% crude fat and 6% crude fiber from listed ingredients. The stock diet and sucrose diet were an 80:20 mixture by weight of this and household sugar.

formula proposed by Moore and Williams (11) for rabbits fed an atherogenic diet.

Livers were homogenized in 0.25 M sucrose and their 105,000 × *g* fat-free supernatants were assayed for soluble protein and G-6-phosphate dehydrogenase activity by methods described previously (12); 6-phosphogluconate dehydrogenase activity was not determined separately.

Aortas were removed from the thorax, briefly inspected for lesions and immersed in ice-cold 0.25 M sucrose. They were then weighed, diced and homogenized [4] for a total of 5 minutes in 2 ml fresh 0.25 M sucrose. The homogenate was washed with 2 ml of the sucrose into a 5-ml Potter-Elvehjem tube and bruised with a loose-fitting Teflon pestle until consistency was judged sufficiently even. Two 2-ml aliquots were centrifuged for 15 minutes at 12,000 × *g* and (without removal) for 60 minutes at 105,000 × *g*, and the volume of fat-free supernatant, recovered carefully with a syringe, was recorded (together with any further additions of 0.25 M sucrose). The supernatant was assayed for protein (13), G-6-phosphate dehydrogenase (12), lactate [5] and alkaline phosphatase (14).

In view of possibilities of initial malabsorption of diet in the refed rats the following tests were performed. A 2.5-cm sample of jejunum was examined and the musculature classified by eye as thick, medium or thin. Distal ileal contents were expressed into a tared beaker, dried at 60° and assayed for glucose, fructose and sucrose by a modification of methods described by Wilson (15), Loewus (16), Smith (17) and others. Cecal contents were expressed into a tared fat-extraction thimble, dried and weighed before and

[4] Using a Sorvall micro-Omnimixer run at an estimated 50,000 rpm.
[5] After incubation with lactic dehydrogenase, using a standard kit (no. 825) obtained from the Sigma Chemical Company, St. Louis.

after ether extraction, in order to estimate their lipid content. In a pilot experiment (unpublished) similar tests had indicated malabsorption at 2 hours and slight malabsorption at 3 hours after first refeeding.

RESULTS

The body weight and intake records are shown in table 2. Differences between the major groups were small. Although the jejunal musculature of the 6-hour refed rats was classified as thin (glucose and fat subgroups) or medium (starch subgroup), no significant malabsorption of fats or sugars at this time was indicated by the tests used. In the starved rats all distal ilea and cecums remained moderately full (the rest of the tract was empty) and the appearance of the contents differed according to the previous diet, suggesting that even after prolonged starvation possibilities of influence by prestarvation diet should not be ignored. The lungs of these rats appeared relatively undamaged by respiratory disease.

Weights and protein content of the livers are shown by groups in table 3. There was a significant overshoot of liver weight 72 hours after refeeding the high carbohydrate diets which was not due to soluble protein and did not occur after refeeding the high fat diet; previous data point towards glycogen accumulation or *de novo* synthesis in the liver (18). The contribution of liver-soluble protein to body weight was unaltered after starvation but was significantly increased, by about 30%, in all groups after refeeding;

TABLE 2

Comparative body weights and average feed intakes

Group (rat nos.)		Body weight, dietary treatment and feed intake [1]					
		Day 0	Day 7	Day 8	Day 9	Day 10	Day 11
1–4	Body wt, % [2]	103	80				
	Starved						
5–8	Body wt, %	99	77	74			
	Refed 6 hr, HG, g [3]			3			
9–12	Body wt, g	98	77	75	80	83	86
	Refed 72 hr, HG, g				14	19	18
13–16	Body wt	101	108	108	108	110	
	Fed ad lib., HG	18	22	19	22	21	
17–20	Body wt	107	79				
	Starved						
21–24	Body wt	106	80	77			
	Refed 6 hr, S			7			
25–28	Body wt	106	79	77	81	86	88
	Refed 72 hr, S				20	22	19
29–32	Body wt	104	113	114	114	116	
	Fed ad lib., CS	24	25	23	25	23	
33–36	Body wt	100	72				
	Starved						
37–40	Body wt	103	74	71			
	Refed 6 hr, HF			5			
41–44	Body wt	98	70	67	72	75	79
	Refed 72 hr, HF				15	15	14
45–48	Body wt	99	108	108	109	111	
	Fed ad lib., C	31	27	25	26	25	

[1] Composition of diets, abbreviations and feeding schedule are described in table 1.
[2] Body weights are expressed as percentages of the group (rats nos. 33–36) fed stock diet ad libitum (average 359 g).
[3] Diets are expressed in grams, being the average intakes for the groups, respectively.

TABLE 3

Weights of livers, and soluble protein content of livers and aortas

Group (rat nos.)	Fed ad libitum [1] Diet [2]		Starved	Refed 6 hours Diet		Refed 72 hours
		Liver weight as percentage [3] of body weight				
		%	%		%	%
1–16	HG	3.45	2.2	HG	2.35	5.47 [4]
17–32	CS	3.5	2.1	S	2.35	4.5 [4]
33–48	C	3.5	2.15	HF	2.25	3.6
		Soluble protein content [5] of livers				
		mg/g	*mg/g*		*mg/g*	*mg/g*
1–16	HG	17	28	HG	28	21
17–32	CS	18	31	S	33	22
33–48	C	18	30	HF	35	25
		Total liver soluble protein as fraction of body weight (mg/100 g)				
		mg/100 g	*mg/100 g*		*mg/100 g*	*mg/100 g*
1–16	HG	59	62	HG	66	96 [6]
17–32	CS	64	65	S	81	100 [6]
33–48	C	63	65	HF	79	91 [6]
		Soluble protein content [7] of aortas				
		mg/g	*mg/g*		*mg/g*	*mg/g*
1–16	HG	8.9	1.9	HG	7.0	5.6 [8]
17–32	CS	10.4	2.9	S	16.5	6.2 [8]
33–48	C	7.5	3.7	HF	13.8	5.5 [9]

[1] Diets and abbreviations as in table 1.
[2] Mean values of subgroups of 4 rats.
[3] Grams/100 g.
[4] Significantly different from ad libitum-fed value ($P < 0.01$).
[5] By the "280/260" method (13). Values in the starved groups were significantly increased ($P < 0.05$ or better).
[6] Significantly higher than the ad libitum-fed values ($P < 0.01$ for the 1–16 group, $P < 0.05$ for the others).
[7] By a method based on that of Lowry (14).
[8] Significantly different from ad libitum-fed value ($P < 0.02$).
[9] Difference from ad libitum fed value not significant ($P < 0.1$).

about half of this increase had occurred by 6 hours except in the high glucose subgroup.

Liver G-6-phosphate dehydrogenase activities (table 4) showed the expected overshoot at 72 hours after refeeding in the high glucose subgroup, and the expected lack of induction in the high fat subgroup. Ad libitum feeding of each of the two very different high carbohydrate diets was associated with 50% increases in activity, whether calculated upon soluble protein or fresh weight. Use of the fresh weight basis showed up some interesting differences, which should be compared with the data in table 3. Although the 6-hour figures show that induction by high glucose diet was somewhat more rapid than by the high starch diet (on either basis), the latter produced considerably higher activity at 72 hours on a fresh weight basis, which became significantly higher if that basis was modified by deduction of liver soluble protein. This point will receive discussion. Furthermore, on a fresh-weight basis liver G-6-phosphate dehydrogenase activity in these rats was neither depressed by starvation nor enhanced by refeeding, per se (table 4, last horizontal line of data).

The blood lipid results (table 5) show significant effects of starvation, of addition of sucrose to the stock diet, of refeeding the starch diet as opposed to the high fat diet, and of feeding or refeeding the high glucose diet, all of these effects being in the direction generally regarded as adverse except that starvation appeared to relieve the highly "adverse" ($P < 0.001$) Moore-Williams ratio in the rats previously fed the high glucose diet. Except during and immediately after starvation, free cholesterol was the main contributor to the alterations in the Moore-Williams

TABLE 4

Liver glucose 6-phosphate dehydrogenase activity

Group (rat nos.)	Fed ad libitum [1] Diet [2]		Starved	Refed 6 hours Diet		Refed 72 hours
		%	%		%	%
		Based on liver soluble protein				
1–16	HG	148 [3] ± 10 [4]	65 ± 7	HG	68 ± 5	844 ± 99
17–32	CS	154 [5] ± 39	63 ± 11	S	48 ± 6	796 ± 48
33–48	C	100 [6] ± 10	58 ± 10	HF	52 ± 5	78 ± 21
		Based on liver fresh weight				
1–16	HG	142 [7] ± 19	101 ± 10	HG	105 ± 9	194 [8] ± 35
17–32	CS	152 [7] ± 21	108 ± 19	S	87 ± 8	262 [8] ± 39
33–48	C	100 [9] ± 10	96 ± 13	HF	103 ± 35	109 ± 38

[1] Diets and abbreviations as in table 1.
[2] Expressed as percentages of the mean values for the subgroup fed stock diet ad libitum.
[3] High significantly above basal value ($P < 0.001$).
[4] SD.
[5] Significantly above basal value ($P < 0.01$).
[6] Represents 59.3 units (18) of G-6-phosphate dehydrogenase activity/mg soluble protein.
[7] Significantly above basal value ($P < 0.02$).
[8] P value for difference between HG and S subgroups < 0.1; but if the weight of soluble protein is deducted from the liver fresh weight and the G-6-phosphate dehydrogenase activity is recalculated on that basis the difference between the HG and S subgroups becomes significant ($P < 0.05$).
[9] Represents 1,063 units (8) of G-6-phosphate dehydrogenase activity per gram of liver. The starved and refed values for this group were not significantly different.

TABLE 5

Plasma cholesterol and phospholipids

Group (rat nos.)	Fed ad libitum Diet [1]	Values [2]	Starved Values	Refed 6 hours Diet	Values	Refed 72 hours Values
		Plasma free cholesterol [3]				
		mg/100 ml	*mg/100 ml*		*mg/100 ml*	*mg/100 ml*
1–16	HG	16 ± 0.8	9 ± 0.5	HG	3 ± 0.5	11 ± 1.3
17–32	CS	7 ± 1.6	14 ± 1.7	S	9 ± 0.8	12 ± 1.3
33–48	C	7 ± 0.9	11 ± 3.1	HF	8 ± 1.3	9 ± 0.7
		Plasma total phospholipids [4]				
		mg/100 ml	*mg/100 ml*		*mg/100 ml*	*mg/100 ml*
1–16	HG	129 ± 7	108 ± 12	HG	51 ± 1	111 ± 30
17–32	CS	124 ± 12	77 ± 35	S	80 ± 14	104 ± 4
33–48	C	131 ± 9	68 ± 3	HF	94 ± 12	124 ± 9
		Moore-Williams ratio (11), % of value for subgroup fed stock diet ad libitum [5]				
		%	%		%	%
1–16	HG	234 ± 20	155 ± 14	HG	123 ± 17	198 ± 34
17–32	CS	110 ± 14	389 ± 87	S	228 ± 40	217 ± 26
33–48	C	100 ± 12	237 ± 56	HF	170 ± 16	144 ± 21

[1] Diets and abbreviations as in table 1.
[2] Mean ± SD of subgroups of 4 rats.
[3] The significant ($P < 0.05$ or better) *vertical* differences are: *ad libitum*: HG group from CS and C; *starved*: HG group from CS; *refed 6 hr*: HG group from S and HF; *refed 72 hr*: none. The significant *horizontal* changes (step by step) are: in *group 1–16*: all 3 steps ($P < 0.01$ or better); *17–32*: all 3 steps; *33–48*: none.
[4] The significant *vertical* differences are: *ad libitum*: none; *starved*: none; *refed 6 hr*: HG from HF; *refed 72 hrs*: S from HF. The significant *horizontal* changes are: *1–16*: all 3 steps; *17–32*: none; *33–48*: by starvation (the other 2 steps were barely significant with $P \cong 0.05$, although at 72 hours the values were significantly above the starved values).
[5] This is the plasma molar ratio of free cholesterol to total phospholipids. In the above test 100% was 0.0267. The significant *vertical* differences are: *ad libitum*: HG group from CS and from C ($P < 0.001$); *starved*: none; *refed 6 hr*: all differences barely significant ($P \cong 0.05$); *refed 72 hr*: CS from C. The significant *horizontal* changes are: *1–16*: by starvation, also the refed 6-hr values differed from the ad libitum-fed values; *17–32*: by starvation; *33–48*: by starvation.

ratio; during starvation the phospholipid side of the ratio appeared to contribute almost equally.

No aortic lesions were seen on brief inspection. A transient overshoot of soluble protein regeneration (table 3) was evident 6 hours after first refeeding, except in the subgroup refed the high glucose diet. Aortic G-6-phosphate dehydrogenase activity (table 6) was abolished by starvation in the high glucose group (no data were obtained from the other groups) and the pattern of activity on refeeding was as follows: at 6 hours there was an overshoot in the high fat subgroup and relatively slow recovery in the high glucose subgroup; at 72 hours the high fat subgroup was normalized, the starch subgroup was returning to normal and the high glucose subgroup had reached a level 71% ($t = 2.933$, $n = 4$; expressed thus for later comparison) of that found when the diet was fed ad libitum. Alkaline phosphatase decreased in the high glucose subgroup fed ad libitum, increased in all groups after starvation and decreased after 72 hours of refeeding, when levels were significantly in descending order of glucose, starch and fat subgroups. Lactate (table 6) showed a marked response to starvation in the group previously fed the high glucose diet, and since the protein-based data given in the footnotes to table 6 show differences from weight-based data similar to differences reported by St. Clair,[6] artifact appears to be unlikely. Comparing the subgroups at 72 hours, the order of values is suggestive but not significant.

[6] Personal communication by R. W. St. Clair, July 13, 1965.

TABLE 6

Aortic glucose 6-phosphate dehydrogenase activity, alkaline phosphatase and lactate

Group (rat nos.)	Fed ad libitum [1] Diet [2]		Starved	Refed 6 hours Diet		Refed 72 hours
		%	%		%	%
G-6-phosphate dehydrogenase activity,[3] compared with rats fed stock diet ad libitum						
1–16	HG	148 [4] ±26 [5]	0	HG	74 [6] ± 15	106 ±26
17–32	CS	192 [7] ±61	— [8]	S	172 ± 25	146 ±109
33–48	C	100 [9] ±34	— [8]	HF	218 ± 89	102 ±27
Alkaline phosphatase,[10] compared with rats fed stock diet ad libitum						
1–16	HG	56 [11] ±14	230 ±148	HG	95 ± 66	20 [12] ±3
17–32	CS	101 ±4	214 ±74	S	66 ± 2	15 [13] ±4
33–48	C	100 [14] ±8	155 ±62	HF	78 ± 61	6 ±4
Lactate,[15] compared with rats fed stock diet ad libitum						
1–16	HG	144 ±71	1,777 [16] ±649	HG	3,208 [17] ±1,075	124 [18] ±53
17–32	CS	78 ±27	13 ±7	S	358 ± 36	109 ±44
33–48	C	100 [19] ±36	40 ±13	HF	302 ± 80	62 ±36

[1] Diets and abbreviations as in table 1.
[2] In each group the percentage refers to the subgroup fed stock diet ad libitum, of which the values are given respectively below.
[3] Based on fresh weight of aortic aliquot, for reasons given in Results and Discussion sections.
[4] Difference from stock diet control subgroup not reckoned significant ($P < 0.1$).
[5] SD.
[6] Significantly ($P < 0.01$) below ad libitum-fed activity of HG fed subgroup.
[7] Significantly ($P < 0.05$) above activity in subgroup fed stock diet ad libitum.
[8] Samples inadvertently destroyed.
[9] G-6-phosphate dehydrogenase activity: 321 units/g of aorta (18).
[10] Based on soluble protein content (table 3) because of the reported association with hypermicrovascularization and because the pattern of results closely resembled that of soluble protein content.
[11] Significantly ($P < 0.05$) below value for subgroup fed stock diet ad libitum.
[12] Significantly ($P < 0.01$) above 72-hour value for high fat refed subgroup.
[13] Significantly ($P < 0.05$) above 72-hour value for high fat refed subgroup.
[14] Alkaline phosphatase value: 36.5 units/mg soluble protein.
[15] Based on fresh weight of aortic aliquot.
[16] The corresponding mean value based on soluble protein was 14,131% of that in the subgroup fed stock diet ad libitum.
[17] The corresponding mean value based on soluble protein was 7,577% of that in the subgroup fed stock diet ad libitum.
[18] The corresponding mean value based on soluble protein was 200% of that in the subgroup fed stock diet ad libitum.
[19] Lactate value: 1.125 μmole/g aorta.

DISCUSSION

Too many variables were present to permit firm conclusions about the data from any but "established" parameters in this experiment, a reservation to be implied throughout the following discussion, but these variables served our exploratory purpose.

Biochemical analysis of human arteries by Kirk (6) using somewhat similar methods indicated that G-6-phosphate dehydrogenase activity in sclerotic portions averaged 74% ($t = 4$, $n = 17$) of that in healthy portions. The similar decrease observed in our high glucose group after starvation and refeeding for 72 hours was perhaps a coincidence. The fact that in the same aortas alkaline phosphatase levels were significantly the highest of the 3 groups was perhaps another coincidence, yet in the subgroup fed high glucose ad libitum, G-6-phosphate dehydrogenase was increased and alkaline phosphatase significantly decreased. Again, this apparent reciprocity was most marked during starvation, but we are unaware of any evidence for a true relationship. The striking effect of starvation on the aortic lactate of the group previously fed high glucose was similar to that produced by St. Clair's experimental hypertension in pigs [7] and may thus be a somewhat unspecific reaction; the overshoot at 6 hours after first refeeding and the comparative levels at 72 hours should also be noted. Taken as a whole these data suggest that the aortic biochemistry of adult male rats is disturbed both by starvation-refeeding and by diets high in glucose, and also that starvation is a major contributor to starvation-refeeding stress. Starvation has been compared, in man, with mild diabetes (19); the atherogenic associations of diabetes are well known (20), and the changes observed in this experiment were consistent with all the associations cited. Furthermore, the presence in the intestines, after prolonged starvation, of matter characteristic of the previous diets supports the suggestion of a continuing influence of those diets.

The importance of cholesterol-phospholipid ratios in the blood has been emphasized by Myasnikow (21) and of *free* cholesterol levels by Leonard et al. (22); the principle in the Moore and Williams formula (11) appears to improve the significance of data which can be recalculated in its terms, e.g., those of Melichar (23), and may deserve wider attention. In our rats an "adverse" blood lipid picture was associated with starvation and with carbohydrate-enrichment of the diet, especially with glucose. An apparent anomaly was the "relief," during starvation, of rats previously fed high glucose.

The liver G-6-phosphate dehydrogenase activities showed the expected "overshoot" at 72 hours after refeeding in the carbohydrate refed groups and not in the group refed the high fat no-carbohydrate diet. There is evidence both for (24) and against (25) the necessity for fasting in order to induce overshoots of this order; in our own test of this point (table 7) fasting contributed significantly. That this G-6-phosphate dehydrogenase overactivity denotes glycogenolysis following a previous overshoot of glycogenesis has been indicated (18), and a contribution of this pathway to hyperlipogenesis has been proposed (26, 27) which would appear capable of accounting for the apparently anomalous "relief," during starvation, of the "adverse" blood lipid picture in our high glucose-fed group. The pattern of refeeding-induction of liver G-6-phosphate dehydrogenase when starch was used differed from that when glucose was used; the change was slower and, at 72 hours, greater; however, the influence of starch on our selected blood lipids and aortic biochemical changes appeared less. As yet we cannot account for such differences. In this experiment dietary protein was not a limiting factor in the induction of G-6-phosphate dehydrogenase (cf. 18). There was no correlation at all between the changes of soluble protein and those of G-6-phosphate dehydrogenase, either in liver or in aorta, although the patterns were respectively consistent. There is some recent confirmation of this,[8] in addition to other positive reasons for preferring to express enzymes regulating carbohydrate metabolism on a basis of liver fresh weight or even body weight (28). The

[7] See footnote 6.

[8] Bergamini, E., G. Bombare and C. Pellegrino 1965 Biochem. J., 97: 1P (abstract).

TABLE 7

Liver glucose 6-phosphate dehydrogenase activity in rats transferred from high fat to high glucose diet without intervening starvation

Group [1]	First diet [2]	Second diet [2]	Treatment	G-6-phosphate dehydrogenase activity [3]			
				Fed ad libitum	Starved	After fed second diet 24 hr	After fed second diet 72 hr
				Units [4]/g of liver fresh weight			
1	HG	—	not starved	9724 ± 703 [5]	—	—	—
2	HF	HG	not starved	1979 ± 421	—	—	5805 ± 649
3	C	HG	starved	—	1899 ± 402	8581 ± 1431	36636 ± 3952
				% of activity in rats fed high fat diet ad libitum			
1	HG	—	not starved	491 ± 36	—	—	—
2	HF	HG	not starved	100 ± 21	—	—	293 ± 33
3	C	HG	starved	—	96 ± 20	434 ± 72	1851 ± 200

[1] Each group contained 5 male Sprague-Dawley rats of 400–500 g.
[2] Diets and abbreviations as in table 1.
[3] Determined by the methods described in the text.
[4] McDonald and Johnson (18).
[5] SD.

movements of soluble protein are themselves interesting (table 3) but we are unable to comment on their relevance to the present experiment.

In this experiment an attempt has been made to explore some possible influences of dietary carbohydrates on pre-lesion biochemical changes in the aortas of adult male rats, using parameters chosen arbitrarily for their reported capacity for change in association with atherosclerosis or hypertension, and also looking for blood and liver changes which could support or fail to support aortic findings. We are not aware of any previous and similar studies. Although these data do not permit firm conclusions to be drawn, their general trend suggests that this type of approach may prove capable of providing useful evidence concerning possible dietary contributions to the etiology of cardiovascular disease.

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Metabolism of Radioactive Phenylalanine in Rats with Different Dietary Intakes of Phenylalanine [1]

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ABSTRACT Diets containing large amounts of phenylalanine are used to induce experimental phenylketonuria in animals and a reduced dietary intake of phenylalanine is an effective control of the mental disease. The metabolism of phenylalanine in rats fed these two different diets was compared with that in normal rats. Following an injection of DL-phenylalanine-3-^{14}C, the phenylketonuric rats excreted more ^{14}C in the CO_2 and urine than normal rats. These values are even higher when DL-phenylalanine-1-^{14}C was used. In both cases more ^{14}C was excreted in the respiratory CO_2 than in the urine. Less ^{14}C, and in particular, less radioactive tyrosine (derived from the injected radioactive phenylalanine), was incorporated into the tissue proteins of the phenylketonuric rats within the first few hours. When rats were maintained with a low phenylalanine diet, less ^{14}C was lost in the CO_2 and urine than with normal rats. The quantity of radioactivity incorporated into the proteins was the same as for normal rats; much less tyrosine-^{14}C was found in animals receiving low phenylalanine diets.

Low phenylalanine diets are prescribed in the treatment of phenylketonuria, and high phenylalanine diets have been shown to be effective in the experimental induction of this condition in rats and monkeys (1, 2). It is important, therefore, to elucidate the metabolism of phenylalanine in animals maintained with these different diets and to compare the results with those observed in normal animals.

EXPERIMENTAL

To induce experimental phenylketonuria, 21-day-old male albino rats of the Wistar strain were fed a 5% L-phenylalanine diet for one week. They were then transferred to a 7% L-phenylalanine diet until the urine gave a positive test when ferric chloride or dinitrophenylhydrazine was added (3). These high phenylalanine diets were prepared by mixing L-phenylalanine with a normal protein test diet.[2]

The phenylalanine-deficient animals were prepared by feeding 200-g male albino rats of the Wistar strain a 0.1% phenylalanine diet [3] until they had lost about 25% of their body weight.

The radioactive compounds used were DL-phenylalanine-3-^{14}C, DL-phenylalanine-1-^{14}C and DL-tyrosine-2-^{14}C (specific activity 1 μCi/mg).[4] The radioactive amino acids were shown to be chromatographically pure, when run on the automatic amino acid analyzer.[5] They were dissolved in isotonic sodium chloride and 0.5 ml containing 1 mg of radioactive amino acid was injected into the left jugular vein. After injection the rats were placed in glass metabolism cages and the respira-

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[2] The protein test diet consisted of the following: (in grams/100 g of diet) casein, 27; starch, 59; vegetable oil, 10; calcium phosphate, 0.45; calcium carbonate, 0.27; calcium citrate, 1.2; cupric sulfate, 0.0003; ferric ammonium citrate, 0.06; magnesium carbonate, 0.14; magnesium sulfate, anhydrous, 0.15; manganese sulfate, 0.0008; potassium chloride, 0.5; potassium iodide, 0.0002; potassium phosphate, dibasic, 0.87; sodium chloride, 0.31; sodium fluoride, 0.002; carotene, 0.15; vitamin D, 0.007; thiamine·HCl, 0.002; riboflavin, 0.002; niacin, 0.06; pyridoxine·HCl, 0.002; Ca pantothenate, 0.004; and α-tocopherol, 0.022. The 5% L-phenylalanine was prepared by mixing 50 g of L-phenylalanine and 950 g of the protein test diet. The 7% L-phenylalanine diet was prepared by mixing 70 g of L-phenylalanine and 930 g of the protein test diet.

[3] The 0.1% phenylalanine diet consisted of the following: (in grams/100 g of diet) pancreatic digest of casein containing amino acids and small peptides processed to remove most of the naturally occurring phenylalanine, 15; starch, 57; corn oil, 18; moisture, 2; DL-methionine, 0.2; L-tyrosine, 0.4; DL-tryptophan, 0.4; calcium as calcium gluconate and calcium hydroxide, 0.65; iron as iron sulfate, 0.01; phosphate as mono and dibasic potassium phosphate 0.5; magnesium as magnesium oxide, 0.1; chlorine as potassium chloride, 0.1; a complete vitamin mixture consisting of the following (in IU/100 g of diet) vitamin A, 1650; vitamin D, 264; and (in mg/100 g of diet) thiamine·HCl, 0.24; riboflavin, 1.2; niacinamide, 0.53; pyridoxine·HCl, 0.4; Ca pantothenate, 2.0; folic acid, 0.03; biotin, 0.02; and vitamin C, 33.0.

[4] The radioactive amino acids were obtained from Calbiochem, Los Angeles, California.

[5] A Technicon Automatic Amino Acid Analyzer was used.

tory CO_2 was collected in a mixture of ethanolamine and ethylene glycol monoethylether (1:2) (4). Food and water were provided during the experimental period. The urine was collected under toluene. To study the incorporation of the radioactive amino acid into proteins the animals were decapitated, the blood was collected over dry potassium oxalate, and the organs were immediately excised. The proteins were obtained from the plasma by precipitation with an equal volume of 10% trichloroacetic acid (TCA). The organs were weighed, homogenized in cold water and centrifuged. The proteins in the clear supernatant were precipitated with TCA.

All radioactivity determinations were carried out in a liquid scintillation counter.[6] Samples of the CO_2 trapping solution or the filtered urine were added to 15 ml of scintillation mixture prepared according to Cuppy and Crevasse (5) and Hayes and Gould (6). The protein samples were dissolved in 1 ml of hydroxide of hyamine, bleached with hydrogen peroxide (7), diluted with 15 ml of the scintillation mixture (8) and counted to a probable error of 2%. Counting efficiency was 45%; the results were corrected for hyamine quenching.

Protein samples were hydrolyzed under vacuum in 6 N HCl at 100° for 20 hours. Quantitative amino acid analyses were performed on an automatic amino acid analyzer.[7]

Urine samples (50 μg) were spotted on Whatman no. 4 paper (15 × 55 cm). A mixture of the pure known substances was also spotted on the same paper strip which was subjected to electrophoresis for 6 hours (300 v, 10 ma). The buffer used was 0.1 M $NaHCO_3$, pH 9.0. After identification of the known markers using ultraviolet light, $FeCl_3$ and ninhydrin, paper squares corresponding to the position of the unknowns were cut out and counted in the liquid scintillation counter.

RESULTS AND DISCUSSION

Excretion of radioactivity in CO_2 and urine

Rats fed high phenylalanine diet. The rats were injected after they had been fed the high phenylalanine diet for at least 2 weeks. Positive ferric chloride and dinitrophenylhydrazine tests indicated that phenylpyruvic acid was being excreted in the urine. All of the rats had large quantities of phenylalanine and even larger quantities of tyrosine in the plasma. Plasma samples from rats maintained with the high phenylalanine diet contained 0.30 ± 0.01 μmole of phenylalanine and 1.66 ± 0.02 μmole of tyrosine /ml. Considering only these biochemical criteria, these animals could be said to be phenylketonuric. Normal rats of the same age had plasma levels of 0.020 μmole of phenylalanine and 0.018 μmole of tyrosine /ml. The plasma samples were obtained from nonfasted rats and food was provided in the metabolism cages to maintain the high phenylalanine and tyrosine plasma levels during the experimental period. It was found that short fasting periods (6 hours) produced a large decrease in the phenylalanine and tyrosine plasma concentration. Pair-fed rats would not have had phenylalanine and tyrosine plasma concentrations that great and therefore were not included in the study.

The quantity of ^{14}C excreted in the respiratory CO_2 and in the urine after the injection of radioactive phenylalanine is shown in table 1. Over the entire 24-hour period all the values for the phenylketonuric rats are higher than those for the normal rats.

After the injection of DL-phenylalanine-3-^{14}C into phenylketonuric rats most of the values are higher than those obtained for normal rats. Except for the 2-hour CO_2 and the 6-hour urine samples, these differences are significant at $P < 0.01$. There was a lag period in the excretion of radioactivity by the phenylketonuric rats. At 2 hours the $^{14}CO_2$ value is the same as for normal rats; by 6 hours it is 3 times as high. This lag period may be the result of dilution of the injected phenylalanine-^{14}C by the large quantities of phenylalanine in the plasma of the phenylketonuric rats.

The high plasma level of tyrosine would also influence the results since DL-phenyl-

[6] Nuclear Chicago Liquid Scintillation Counter, model no. 8401, system 703.
[7] See footnote 5.

TABLE 1

Excretion of radioactivity in CO_2 and urine of rats injected with radioactive phenylalanine

Group [1]	Injection [2]	Recovered in CO_2, hours				Recovered in urine, hours			
		2	6	12	24	6	12	24
Normal	DL-Phe-3-^{14}C	2.85 ± 0.90 [3]	5.64 ± 1.47	7.17 ± 1.87	9.55 ± 2.07	2.15 ± 0.81	2.79 ± 1.09	3.51 ± 1.02
High Phe diet	DL-Phe-3-^{14}C	2.63 ± 0.87	17.33 ± 3.63	24.22 ± 4.12	25.77 ± 3.62	2.38 ± 1.49	4.86 ± 1.90	7.37 ± 2.36
Low Phe diet	DL-Phe-3-^{14}C	1.99 ± 0.78	3.72 ± 1.49	5.03 ± 1.54	6.91 ± 1.78	1.15 ± 0.45	1.62 ± 0.49	2.08 ± 0.69
Normal	DL-Phe-1-^{14}C	5.66 ± 1.03	9.75 ± 1.15	11.38 ± 1.22	13.78 ± 2.02	3.01 ± 1.07	3.38 ± 0.90	3.69 ± 0.86
High Phe diet	DL-Phe-1-^{14}C	9.58 ± 2.58	33.09 ± 2.94	41.20 ± 4.90	42.20 ± 4.53	8.50 ± 1.90	10.42 ± 3.24	11.86 ± 3.82
High Phe diet	DL-Phe-1-^{14}C + methyl-DOPA [4]	6.87 ± 2.40	25.89 ± 10.68	33.00 ± 9.90	39.07 ± 8.66	5.85 ± 1.30	7.10 ± 1.69	7.03 ± 1.89
Normal	DL-Tyr-2-^{14}C	17.00 ± 1.32	25.48 ± 2.45	27.53 ± 2.28	31.16 ± 2.16	6.27 ± 1.30	6.82 ± 1.51	7.76 ± 1.60
High Phe diet	DL-Tyr-2-^{14}C	16.57 ± 1.53	37.33 ± 3.20	47.64 ± 2.61	49.84 ± 4.10	8.06 ± 1.12	12.02 ± 2.10	14.12 ± 1.89

[1] Eight rats/group.
[2] Phe: phenylalanine; Tyr: tyrosine.
[3] Averages ± SE of mean of cumulative percentages of administered dose excreted.
[4] DL-α-Methyl-DOPA (20 mg in 0.5 ml isotonic NaCl) was injected intraperitoneally one-half hour before administration of the radioactive substance.

alanine-3-^{14}C must be transformed to tyrosine before any $^{14}CO_2$ is produced (at the level of the Krebs cycle). The higher ^{14}C content of the urine is probably a reflection of the increased urinary excretion of phenylalanine, phenylpyruvic acid and other phenylalanine metabolites, characteristic of phenylketonuria. Feces were homogenized in water and aliquots were counted in the liquid scintillation counter. Only traces of radioactivity were detected. DL-Tyrosine-2-^{14}C was also injected into normal and phenylketonuric rats. Most of the values for the phenylketonuric rats are higher than those obtained for normal rats (table 1). There was therefore an acceleration of the tyrosine catabolism under these conditions.

The ^{14}C excretion in the CO_2 and urine was also increased over that of normal rats when DL-phenylalanine-1-^{14}C was injected. In 24 hours, 42% of the injected dose was expired in the CO_2. This $^{14}CO_2$ can be produced at four main sites: 1) when phenylalanine is converted to phenylethylamine; 2) during the formation of phenylacetic acid from phenylpyruvic acid; 3) by decarboxylation of tyrosine or its derivatives such as DOPA; and 4) after transformation into tyrosine, at the level of *p*-hydroxyphenylpyruvic acid. The first 3 sites involve straight decarboxylation of the aromatic compound, a process which is inhibited by α-methyl-DOPA (9). The last site of $^{14}CO_2$ production involves oxidative decarboxylation. When α-methyl-DOPA was injected one-half hour before the radioactive compound, there was a decrease of only 30% in the $^{14}CO_2$ after 2 hours (table 1) and this decrease is not significant. That is, in the phenylketonuric rats more than two-thirds of the radioactive phenylalanine was still metabolized via the tyrosine pathway. The small quantities of radioactivity excreted in the urine also indicates that the formation of phenylpyruvic acid is not the main catabolic route.

When DL-phenylalanine-1-^{14}C is used, it would be expected that less radioactivity would be excreted in the urine than when the phenylalanine is labeled on the third carbon; phenylethylamine, phenyllactic acid and phenylacetic acid, all of which are present in the urine, would not be radioac-

TABLE 2

Distribution of radioactivity in urine of rats injected with radioactive phenylalanine

Group [1]	Injection	Duration	Urine fractions [2]					
			HPA	PA	Tyr	Phe	Urea	PNH_2
		hours	*% of total radioactivity in urine*					
Normal	DL-Phe-3-^{14}C	6	2.8	11.3	28.0	35.9	4.2	18.7
Normal	DL-Phe-3-^{14}C	12	0.6	9.6	21.3	34.2	2.2	30.0
Normal	DL-Phe-3-^{14}C	24	0.0	3.5	18.4	13.5	3.5	60.1
Normal	DL-Phe-1-^{14}C	6	3.8	7.6	31.0	41.5	8.4	0.0
Normal	DL-Phe-1-^{14}C	12	5.3	4.4	22.1	22.2	46.0	0.0
Normal	DL-Phe-1-^{14}C	24	4.3	2.5	12.2	16.3	61.7	0.0
High Phe diet	DL-Phe-3-^{14}C	6	7.9	17.2	28.3	42.5	1.5	1.2
High Phe diet	DL-Phe-3-^{14}C	12	7.6	18.9	27.1	34.5	6.6	3.2
High Phe-diet	DL-Phe-3-^{14}C	24	0.0	3.6	19.8	30.2	4.6	40.1
High Phe diet	DL-Phe-1-^{14}C	6	4.0	7.8	27.8	52.3	5.6	0.3
High Phe diet	DL-Phe-1-^{14}C	12	6.5	6.9	24.6	34.0	25.6	0.2
High Phe diet	DL-Phe-1-^{14}C	24	0.0	0.6	12.9	23.8	58.7	1.7

[1] Four rats/group, urine samples were pooled.
[2] HPA: p-hydroxyphenylpyruvic and p-hydroxyphenyllactic acids. PA: phenylpyruvic and phenyllactic acids. Tyr: tyrosine. Phe: phenylalanine. PNH_2: amines derived from aromatic amino acids.

tive. However, (table 1) in normal rats there was slightly more ^{14}C in the urine when DL-phenylalanine-1-^{14}C was used (table 1). This level was greatly elevated in the phenylketonuric rats. When the urine was separated by paper electrophoresis at pH 9.0 several radioactive fractions were obtained (table 2). In order of decreasing mobility toward the cathode, the following fractions were identified: a fraction containing *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid; a fraction containing phenylpyruvic and phenyllactic acid; and tyrosine and phenylalanine. Moving toward the anode, urea and a fraction containing the amines derived from aromatic amino acids (PNH_2) were identified. The distribution of radioactivity in these different fractions is shown in table 2. Tyrosine and phenylalanine are the two most important radioactive compounds in 6- and 12-hour urine samples. There was a slight increase in phenylalanine excretion in the phenylketonuric rats. The keto and hydroxy acids were increased in the phenylketonuric rats but they remain minor metabolites of aromatic amino acid catabolism. The increase in *p*-hydroxyphenyl-keto and hydroxy acids in the phenylketonuric rats is an indication that these rats were also in a state of tryosyluria (10). In the urine obtained after injection of DL-phenylalanine-3-^{14}C, the amine fraction was strongly radioactive, especially after 24 hours, whereas in urine obtained after injection of DL-phenylalanine-1-^{14}C, urea was strongly radioactive. Since more radioactive CO_2 was produced from DL-phenylalanine-1-^{14}C, it is possible that some of the $^{14}CO_2$ was used in the synthesis of urea. This could explain the increased urinary excretion of radioactivity when DL-phenylalanine-1-^{14}C was used. Urea was extracted and crystallized out of the urine of the phenylketonuric rats that had been injected with DL-phenylalanine-3-^{14}C, DL-phenylalanine-1-^{14}C and DL-phenylalanine-1-^{14}C plus methyl-DOPA (11). The specific activity of the urea was 28, 748 and 648 cpm, respectively. The CO_2 produced by oxidative decarboxylation of phenylalanine is more available for the synthesis of urea than CO_2 produced at a later stage of catabolism.

Rats fed a low phenylalanine diet. These rats were tested only with DL-phenylalanine-3-^{14}C. All values for the ^{14}C content of the CO_2 and urine were lower than those for normal rats (table 1). These differences became significant only at 6 hours ($P < 0.01$).

Distribution of radioactivity in tissue proteins

The quantities of radioactive amino acid which were incorporated into the proteins are shown in table 3. When normal rats were injected with DL-phenylalanine-3-^{14}C, the highest level of incorporation was in the pancreas proteins at the 2-hour period. Over the 24-hour experimental period, the quantity of radioactivity in the

TABLE 3

Distribution of radioactivity in solubilized tissue proteins of rats injected with radioactive phenylalanine

Group [1]	Injection [2]	Duration	Tissue				
			Liver	Kidney	Pancreas	Brain	Plasma
		hours	*cpm/mg*	*cpm/mg*	*cpm/mg*	*cpm/mg*	*cpm/mg*
Normal	DL-Phe-3-^{14}C	2	32.0 ± 3.4 [3]	77.3 ± 5.6	343.1 ± 21.0	17.4 ± 0.5	85.1 ± 1.4
Normal	DL-Phe-3-^{14}C	24	33.8 ± 4.5	99.6 ± 6.1	38.2 ± 4.7	14.5 ± 1.2	65.1 ± 1.3
High Phe diet	DL-Phe-3-^{14}C	2	23.9 ± 1.8	33.3 ± 1.8	118.5 ± 4.7	12.0 ± 0.6	46.9 ± 2.8
High Phe diet	DL-Phe-3-^{14}C	24	14.1 ± 2.3	29.4 ± 1.2	19.2 ± 1.4	14.1 ± 0.5	30.4 ± 1.6
Low Phe diet	DL-Phe-3-^{14}C	2	47.0 ± 8.7	112.4 ± 8.4	334.0 ± 11.1	21.5 ± 4.5	74.7 ± 1.51
Low Phe diet	DL-Phe-3-^{14}C	24	48.4 ± 7.5	96.6 ± 7.1	76.3 ± 6.5	29.1 ± 3.6	82.9 ± 2.55

[1] Eight rats/group.
[2] Phe: phenylalanine.
[3] Mean ± SE.

liver proteins was rather constant. In brain and plasma proteins the level decreased slowly. In the pancreas proteins there was a rapid decrease, whereas the kidney proteins showed a gradual decrease with time.

In rats maintained with the high phenylalanine diet, less radioactivity was incorporated into the proteins of all the organs. The pancreas proteins, at 2 hours, have the highest values. The radioactivity of all of the proteins decreased with time.

When the rats that had been maintained with a low phenylalanine diet were injected with DL-phenylalanine-3-^{14}C, the level of incorporation of radioactive amino acids into tissue proteins was generally higher than that obtained in normal rats. However, the radioactivity in the pancreas and plasma proteins after 2 hours was slightly decreased.

These results indicate that, in rats fed the high phenylalanine diet, the injected radioactive phenylalanine was diluted by the excess plasma phenylalanine. Therefore, less ^{14}C was incorporated into the proteins. In rats that were deprived of phenylalanine, the radioactive amino acid was used for protein synthesis slightly more efficiently than in normal rats.

It is known that injected radioactive phenylalanine is transformed into radioactive tyrosine which is also incorporated into protein (12). This radioactive tyrosine is metabolized differently from injected radioactive tyrosine (13). Radioactive proteins, obtained after injection of radioactive phenylalanine, were hydrolyzed and analyzed. The ratios of the radioactivity of the isolated tyrosine to the isolated phenylalanine are given in table 4. In normal rats, the ratios are comparable to those shown for the plasma proteins of dogs (12). Between 20 and 25% of the radioactivity in the proteins was in the form of tyrosine.

In phenylketonuric rats, the ratios are much lower (2 hours after injection). By 24 hours, the ratios are comparable to, and even higher than, those for normal rats. This may mean that in the phenylketonuric rats the rate of transformation of injected phenylalanine into tyrosine was slower than in normal rats. It is also possible that the rate of transformation

TABLE 4

Ratios of radioactive tyrosine to radioactive phenylalanine

Group [1]	Pancreas proteins		Plasma proteins	
	2 hours	24 hours	2 hours	24 hours
Normal rats	0.15 [2]	0.22	0.28	0.29
Fed high Phe diet	0.03	0.53	0.13	0.25
Fed low Phe diet	0.08	0.67	0.09	0.38

[1] Four rats/group.
[2] Ratio of the total radioactivity present in the tyrosine peak to that in the phenylalanine peak. These peaks were obtained from a protein hydrolysate separated on the automatic amino acid analyzer.

was the same or faster but that the large quantity of nonradioactive tyrosine already present, as a result of the diet, diluted the radioactive tyrosine. Therefore, less radioactive tyrosine was incorporated into protein. The second assumption appears to be more likely. The higher $^{14}CO_2$ production does not indicate that there was any decrease in the rate of the hydroxylation of phenylalanine to tyrosine in the phenylketonuric rats. Also, the ratios obtained in the phenylketonuric rats are much higher than those from phenylketonuric patients (11). In the latter case the liver phenylalanine hydroxylase was relatively inactive.

In the phenylalanine-deficient rats, the ratios are also low 2 hours after injection. However, by 24 hours they are greatly increased. This indicates that, at first, most of the radioactivity in the proteins was caused by the incorporation of adioactive phenylalanine. Later, a large quantity of radioactive tyrosine was produced and incorporated into proteins.

CONCLUSIONS

When rats were fed a high phenylalanine diet for 2 weeks, high levels of phenylalanine and tyrosine are found in the plasma. Phenylpyruvic acid was excreted in the urine. Injection of radioactive phenylalanine into these rats produced a decrease in the incorporation, into tissue proteins, of radioactive phenylalanine and radioactive tyrosine (derived from the injected phenylalanine) compared with the results from normal rats. Therefore, more phenylalanine and tyrosine were catabolized, as shown by the increased radioactivity in the CO_2 and urine. However, the loss of ^{14}C via the urine was relatively small. This indicates that the catabolism of phenylalanine via phenylpyruvic acid and its derivatives is not of major importance in the phenylketonuric rats, and that metabolically, phenylketonuric rats are quite different from phenylketonuric humans. It also suggests that most of the D-phenylalanine is efficiently used by the rat. In rats D- and DL-phenylalanine are almost as satisfactory for maintaining maximal growth as L-phenylalanine (14).

It would be expected that the rats maintained with a low phenylalanine diet for some time would specifically require this amino acid. In fact, after the injection of phenylalanine-^{14}C less radioactivity was lost via the CO_2 and urine than in normal rats and the amount of ^{14}C incorporated into the tissue proteins was increased. The main difference, however, is that, 2 hours after injection, most of the radioactivity found in the proteins of the phenylalanine-deficient animal was in the form of radioactive phenylalanine. In normal rats, 20 to 25% of the radioactivity was in the form of radioactive tyrosine. This larger quantity of radioactive phenylalanine, incorporated into the proteins of the phenylalanine-deficient rat, is probably a reflection of the great need for the essential amino acid for protein synthesis.

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Amino Acids in Postprandial Gut Contents of Man [1]

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ABSTRACT The objective of the investigation was to determine the free amino acids in the lower duodenum of man 1.5 hours after ingestion of food. The 4 types of test meals consisted of lean beef, gelatin, eggs or milk. Subjects were 5 healthy males 21 to 64 years of age. Molar ratios of amino acids in the doudenal contents were different from those derivable from hydrolysis of the test meal proteins in vitro. For example, after gelatin ingestion, the amount of tryptophan present was as great as when the complete proteins of eggs and milk were ingested. It is concluded that large quantities of endogenous protein enter the duodenum of man during digestion and are mixed with ingested protein. On hydrolysis, this protein mixture yields an amino acid mixture for absorption which is quite different from that provided by the ingested protein alone.

A decade ago, Nasset et al. (1) demonstrated in the dog that ingestion of distinctly different test meals (egg albumin, zein, non-protein) yielded molar ratios of free amino acids in gut contents that were relatively constant. These results were interpreted to mean that large quantities of endogenous protein (enzymes, secretions, mucosal cells) entered the gut lumen and tended to obscure the unique amino acid composition of ingested protein. This type of amino acid homeostasis in the gut lumen has not been demonstrated in man. The experiments described below were made to provide some data on this subject.

METHODS

The subjects were 5 healthy, postabsorptive, human males aged 21, 22, 33, 39 and 64 years. In the morning a duodenal tube with slotted metal tip was swallowed and by the usual maneuvers worked into the duodenum in 2 to 2.5 hours. The tip was usually at the ligament of Treitz or 2- to 3-cm distal to it, as demonstrated by fluoroscopic examination.

With the tube in place the test meal was ingested. The 4 types of test meals consisted of lean beef, gelatin, eggs or milk. Before ingestion: 200 g lean beef muscle (round steak) were minced and broiled; 18 g of ossein gelatin [2] were dissolved in 400 ml of hot water and allowed to gel; 3 medium-size hen's eggs were boiled for 10 minutes; 450 ml of whole, homogenized milk (3.5% fat) were chilled. Water 200 ml, and salt were taken with meat and egg meals. These 4 types of meals were chosen because they are foods that are not uncommon in self-selected diets and they represent considerable variation in their content of amino acids. Subject O ate all four test meals; subject N ate three; subjects H and T ate two each; and subject K ate one. No subject ate the same test meal twice.

Gut contents were withdrawn by syringe 1.5 hours after ingestion was completed, heated at 95° for 15 minutes to inactivate enzymes and kept frozen until required for analysis. Moisture was determined by drying to constant weight at 115°; ash by heating at 490° in a muffle furnace; total N by micro-Kjeldahl; NPN by micro-Kjeldahl on the filtrate from picric acid deproteinization (excess picrate removed by Bio-Rad Resin AG1 - x 8, 200-400 mesh, chloride form); amino acids on picric acid filtrates according to Spackman et al. (2).

RESULTS

The results of proximate analyses plus computation of the fraction of N represented by NPN and amino acid N are shown in table 1. Amino acid analyses are shown in table 2. The accelerated analysis

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[2] A gift from Knox Gelatine Inc., Camden, New Jersey.

TABLE 1

Proximate analyses of gut contents obtained 1.5 hours after ingestion of test meals by fasting man

Test meal	Moisture	Solids	Ash	Total N	NPN	NPN × 100 / Total N	Amino acid × 100 / NPN
	%	%	%	*g/100 ml contents*			
Beef muscle	95.5 ± 0.7 [1]	4.6 ± 0.7	0.7 ± 0.05	0.45 ± 0.09	0.22 ± 0.03	49	18
Gelatin	98.2 ± 0.2	1.8 ± 0.3	0.7 ± 0.05	0.15 ± 0.03	0.13 ± 0.03	87	11
Egg	95.5 ± 0.7	4.6 ± 0.8	0.7 ± 0.10	0.36 ± 0.03	0.13 ± 0.01	36	16
Milk	95.9 ± 0.2	4.1 ± 0.3	0.6 ± 0.10	0.20 ± 0.02	0.12 ± 0.01	60	7.5

[1] Mean ± SE of 3 samples, obtained from different subjects.

used for these determinations failed to give satisfactory results for 1/2 cystine and proline. Some proline apparently was lost during removal of excess picrate from the filtrates used for amino acid analysis. Table 3 contains molar ratios of amino acids in the proteins as ingested and in the duodenal contents resulting from their ingestion.

DISCUSSION

As indicated in table 1 the duodenal contents were quite watery. The gelatin and milk meals contained at least 400 ml of water. This is reflected in the total N of the contents as compared with the contents derived from beef and eggs. The NPN values for 3 of the meals are the same, but for the beef meal the concentration is about 50% greater probably owing in part to the larger amount of total N ingested. Gelatin yielded the greatest proportion of NPN but since the amino acid N is relatively low it seems probable that peptide N was high 1.5 hours after the gelatin meal. However, partial digestion of all proteins had proceeded to a considerable extent even at a point in the gut just distal to the pancreatic ducts. Table 2 shows that the beef meal yielded the greatest quantities of all individual amino acids determined, and that the total was 2 to 4 times as great as for other meals. This is due in part at least to the larger mass of the meat meal.

In the original paper on this subject (1), dealing with experiments on dogs, it was demonstrated that molar ratios of free amino acids in jejunal and ileal contents were relatively constant regardless of the test meal fed. Duodenal contents were not modified as much but enough to make it difficult if not impossible to determine, from amino acid analyses, whether egg albumin, zein or a non-protein meal had been fed. A similar condition appears in man as is evident from data in table 2. Gelatin, e.g., contains no tryptophan but duodenal contents derived from gelatin ingestion contain as much tryptophan as the contents derived from the other test meals. Looking at the data in table 2 can be confusing owing to the great differences in concentration of amino acids per 100 ml. A better comparison can be made as in table 3 where molar ratios are used.

Gelatin is given a detailed discussion based on table 3 because it is an incomplete protein. Beef muscle, eggs and milk all yield proteins of high biological value and would not yield the striking results obtained with gelatin. It is evident, however, that even the complete proteins yield amino acid mixtures in the duodenum that can be quite different from those derivable from hydrolysis of the proteins in vitro. In table 3, the data indicate that after gelatin ingestion the duodenal contents contain an amino acid mixture in which the molar ratios of all of the essential amino acids have been increased. The most striking change is in tryptophan. All of the nonessential amino acids determined, except tyrosine and serine, moved in the opposite direction. In duodenal contents derived from all test meals the molar ratios of arginine, lysine, methionine and phenylalanine were all increased and those of alanine, aspartic and glutamic acids were all decreased.

The amino acid pool found in the gut lumen during digestion is composed of contributions from both endogenous and exogenous sources. Estimates of the relative magnitudes of these contributions were presented in a previous paper (3)

TABLE 2

Free amino acids obtained from gut contents 1.5 hours after ingestion of test meals by fasting man

Test meal	Free amino acids																Total
	Ala	Arg	Asp	Glu	Gly	His	Ileu	Leu	Lys	Met	Phe	Ser	Thr	Try	Tyr	Val	
	µmoles/100 ml contents											*µmoles/100 ml contents*					
Beef muscle	84.0	161.2	34.5	73.5	66.2	229.0	45.6	172.0	357.0	72.5	106.4	58.8	42.4	35.0	95.9	113.5	1748
	± 6.4 [1]	±18.4	±24.0	±24.8	±10.1	±33.4	± 5.4	± 4.4	±52.6	± 5.6	±20.3	± 5.6	±9.0	±18.8	± 6.1	± 3.5	
Gelatin	51.0	69.6	12.7	12.3	72.5	14.3	26.2	55.2	86.0	19.6	27.4	32.8	20.3	15.0	30.0	56.8	602
	±16.8	± 6.2	± 5.0	± 4.9	±28.0	± 4.4	± 4.7	± 1.5	±11.6	± 4.2	± 9.6	± 8.3	±5.8	± 3.8	±11.5	± 8.3	
Egg	52.5	113.3	17.0	53.0	25.8	5.9	30.7	152.4	105.9	57.7	95.1	44.8	30.9	10.0	66.1	60.6	922
	±12.9	±26.6	± 2.8	± 9.0	± 5.1	± 1.8	± 6.3	±22.6	±28.3	±15.2	±19.5	±10.6	±5.8	± 1.4	± 9.1	± 5.8	
Milk	12.3	37.8	14.9	32.4	15.4	6.1	12.5	29.4	80.2	22.5	26.3	19.6	8.6	10.8	39.5	19.8	388
	± 4.0	±10.5	±13.4	± 9.8	± 3.6	± 1.1	± 0.7	± 7.2	±33.0	± 2.2	± 5.7	± 4.3	±2.4	± 1.8	± 8.6	± 8.9	

[1] Means ± SE of 3 samples, obtained from different subjects.

TABLE 3

Moles of individual amino acids per 1000 moles of total amino acids as determined in gut contents and in test meal proteins as ingested[1]

		Ala	Arg	Asp	Glu	Gly	His	Ileu	Leu	Lys	Met	Phe	Ser	Thr	Try	Tyr	Val
Beef	contents	48.1	92.2	19.7	42.1	38.4	131.0	26.1	98.3	204.0	41.5	60.8	33.7	24.3	20.1	54.8	64.8
	protein	102.5	53.2	84.4	139.4	93.8	25.3	50.4	85.6	81.8	23.5	38.3	58.3	54.3	7.6	24.1	66.0
Gelatin	contents	84.6	115.5	21.1	20.4	120.2	23.7	43.5	91.3	142.7	32.5	45.5	54.4	33.7	24.9	49.8	94.3
	protein	150.0	61.7	60.0	93.5	431.0	5.4	13.9	31.3	35.5	5.2	18.0	42.2	23.6	0.0	1.5	28.2
Egg	contents	57.0	123.2	18.5	57.5	28.0	6.4	33.3	165.3	115.0	62.5	103.0	48.6	33.5	10.8	71.6	65.7
	protein	101.3	50.2	80.5	112.0	62.6	20.2	69.8	93.6	61.6	28.9	45.9	96.9	54.7	10.2	29.5	82.5
Milk	contents	31.7	97.5	38.4	83.6	39.7	15.7	32.3	75.8	207.0	58.1	67.8	50.6	22.2	27.9	102.0	51.1
	protein	56.4	28.0	78.6	206.0	39.1	24.3	69.1	105.2	76.3	22.5	43.0	69.0	55.0	8.9	37.7	79.7

[1] Amino acid composition of protein obtained from Amino Acid Handbook, eds., R. J. Block and K. W. Weiss 1956 Charles C Thomas, Springfield, Illinois.

and it was demonstrated that the endogenous contributions may outweigh the exogenous ones by several-fold. These observations on animals have now been confirmed in part by the present observations on man. It will be interesting to determine the amino acid composition of jejunal and ileal contents in man.

Judging from the data for moisture and total N (table 1), as well as amino acid concentrations (table 2), the digestive apparatus may respond differently to different test meals. This is a subject that has not been systematically investigated. In acute experiments, such as those described above, any marked change in secretory response of the digestive glands might conceivably cause a marked change in the amount and kind of protein secreted as enzymes and mucus. A meal composed of crystalline amino acids, e.g., would certainly exert an immediate osmotic effect, not characteristic of normal foods and could very well exert an atypical stimulatory effect on the digestive glands. These are problems that require further investigation before it is possible fully to evaluate responses to ingestion of single meals such as were used in the present study as well as those made up of crystalline amino acids and other compounds that are normally the end products of digestion. It is obvious that sustained subsistence with a diet containing an incomplete protein or amino acid mixture may induce responses which may not necessarily be manifest after ingestion by an adequately nourished animal, of a single test meal of the same diet.

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Utilization of Environmentally Produced High Nitrogen Corn by Weanling Rats and Adult Humans [1]

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ABSTRACT Experiments with rats and human subjects were carried out to study the utilization of nitrogen from high-nitrogen corn produced by natural or artificial defoliation of the corn plant at various stages of development. Weight gain, food intake and digestible nitrogen were significantly higher and food required per unit of gain was significantly lower for rats fed high-nitrogen corn. That the increased protein content of the damaged corn was not due to an increase in zein content, was demonstrated by feeding zein in combination with control corn. Nitrogen balances of human subjects were studied when control corn, corn defoliated at the milk stage, and corn defoliated at the blister stage were fed in isonitrogenous amounts. No significant differences were observed in response of subjects to the 3 types of diet; however, nitrogen balance tended to be higher and urinary nitrogen lower when the milk-stage corn was fed. Amino acid analyses indicated that the increase in essential amino acids in the high-nitrogen corn was proportional to the increase in nitrogen content.

Corn is considered a poor source of dietary protein because of its low total protein content and its high content of zein, and because it is deficient in lysine and tryptophan. Efforts have been made to increase the protein content of corn by genetic or environmental modification; however, in the early work high protein corn was found to have higher zein content (1) and a lower biological value (2, 3) when compared with regular corn.

Hail, frost and drought cause appreciable damage to corn annually. Damaged corn has been found to have a higher protein content than regular (undamaged) corn.[2] Since the nature of the increase in protein was unknown, it seemed worthwhile to determine whether the increase in nitrogen content improved the quality of protein of the corn.

Hail and frost damage may be simulated by physical or chemical defoliation of the plant at different stages of the growth period. In general, complete defoliation of the plant produces corn with the highest protein content.[3]

The objective of the present investigation was to determine the value of high-nitrogen corn for support of growth in rats and maintenance of nitrogen balance in adult human subjects.

RAT STUDY

In the first study, the nutritive quality of various types of high-nitrogen corn was compared with that of control corn (unaltered) and with control corn made isonitrogenous with high-nitrogen corn by the addition of zein. Growth rate, feed intake, feed efficiency, digestible energy, dry matter, and nitrogen were used as criteria of response.

PROCEDURE

In this study, zein was added to bring the nitrogen content of control corn up to that of the damaged corn.

Crossbred Norway rats (domestic strain) were caged individually in a constant-temperature-humidity room. The 24 rats were divided at random into 6 groups of 4 rats each. Experimental rations were fed ad libitum.

Dietary protein was supplied by 6 types of corn from the same genetic stock produced in 1962. Composition of the rations is shown in table 1. A constant percentage

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[2] Colville, W. L. 1964 Personal communication. Nebr. Agr. Exp. Sta., Lincoln, Nebraska.

[3] See footnote 2.

TABLE 1

Composition of experimental ration for rat study

	%
Ground yellow corn	94.7
Salt (iodized)	0.4
Ground limestone	1.7
Monosodium phosphate	1.1
Trace mineral mix [1]	0.1
Vitamin premix [2]	2.0
Protein (chemical analysis) [3]	variable
Calcium (calculated)	0.65
Phosphorus (calculated)	0.66

[1] Percentage composition: Mn, 10.0; Fe, 10.0; Cu, 1.0; Co, 0.10; I, 0.30; Zn, 10.0 and Ca, 9.1; obtained from Calcium Carbonate Company, Quincy, Illinois.
[2] Vitamin premix contributed the following per kg feed: vitamin A (vitamin A palmitate 125,000 IU/g), 5280 IU; vitamin D_2, 990 IU; vitamin B_{12}, 33 μg; and (in milligrams) riboflavin, 4.4; niacin, 6.6; pantothenic acid, 13.2; choline chloride, 998.8; α-tocopheryl acetate, 44.0; menadione, 2.2; thiamine·HCl, 2.2; and pyridoxine·HCl, 2.2.
[3] Ration 1, 9.7% (control corn); ration 2, 9.7% (control corn) plus 3.5% (zein); ration 3, 10.9% (frosted corn); ration 4, 10.9% (frosted corn) plus 2% (zein); ration 5, 12.0% (frosted corn); and ration 6, 12.3% (artificially defoliated corn).

of corn as raw cornmeal (approximately 95%) was included in the ration. Since the types of corn varied in protein content, the percentage of protein from corn varied as follows: ration 1: 9.7% protein from control corn; ration 2: 9.7% protein from control corn plus 3.5% protein from zein; ration 3: 10.9% protein from corn damaged by natural frosting at the dough stage; ration 4: 10.9% protein corn damaged by frost at the dough stage plus 2% protein from zein; ration 5: 12% protein from corn damaged by frost at the milk stage; and ration 6: 12.3% protein from corn artificially defoliated at the milk stage of development.

Weight gain and feed intake were recorded weekly. The feces were collected twice daily and immediately frozen in tightly sealed plastic bags until they could be processed. Nitrogen content of the various types of corn as well as of the excreta was determined by the Kjeldahl method. Energy content of ration and excreta was determined in a bomb calorimeter. Dry-matter content of ration and feces was also determined by a modification of the procedure outlined by AOAC (4).

RESULTS

The growth responses of rats fed the 6 experimental rations are shown in figure 1. The rats averaged 50 g at the beginning of the experiment. Total weight gains over the 36 days were: 42 g (control corn plus zein); 49 g (control corn); 84 g (frost-damaged corn); 85 g (frost-damaged corn plus zein); 100 g (frost-damaged corn at the milk stage); and 106 g (artificially defoliated corn).

Data relating to daily feed intake, feed per gram of gain, digestible energy, digestible dry matter and digestible nitrogen are shown in table 2. Statistical comparisons were made between rations containing control corn and those containing damaged corn. Weight gain, food intake and digestible nitrogen were significantly higher ($P < 0.01$) and food required per unit of gain was significantly lower ($P < 0.01$) for rats fed damaged corn.

No differences were found between digestible energy and digestible dry matter for rats fed the 2 types of rations.

Digestible energy paralleled digestible dry matter, and digestible nitrogen was lowered by addition of zein to either control (ration 1 vs. ration 2) or damaged corn (ration 3 vs. ration 4).

HUMAN STUDY

Because the study with rats showed that the increase in nitrogen content of damaged or defoliated corn did not result from an increase in zein content, microbiologi-

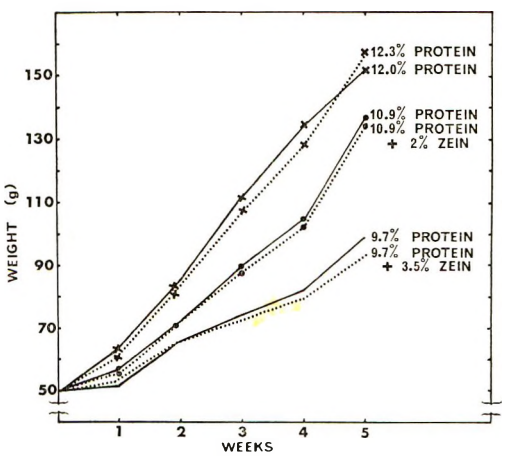

Fig. 1 Growth of rats fed control and damaged corn. Legend: ——— control corn; ..... control corn plus zein; ●———● dough stage (natural); ●.....● dough stage plus zein; ×———× milk stage (natural); ×.....× milk stage (artificial).

cal analysis of amino acids in certain of the altered types of corn was performed. Essential amino acids, except tryptophan, were determined in control corn, corn defoliated at the milk stage and corn defoliated at the blister stage. The method used in determination of amino acids in corn was as described by Steele (5). Amino acid analyses indicate an overall increase in essential amino acid content of defoliated corn over that of the control corn. When amino acid content was calculated on the basis of milligrams of amino acid per gram of nitrogen, a fairly constant figure was observed among the 3 types of corn (table 3).

Since the increase in essential amino acids was proportional to the increase in total nitrogen of corn, it seemed reasonable to assume that feeding equal amounts of nitrogen from regular (undamaged) and damaged corn would result in a similar response in a biological evaluation. Accordingly, a nitrogen balance study was planned in which human subjects were fed a constant level of nitrogen from the various types of corn.

The study was composed of a 3-day depletion period, a 7-day adjustment period and 3 experimental periods of 5 days each, arranged at random.

PROCEDURE

Subjects: Six adult volunteers, described in table 4, participated as subjects. All were considered to be in good health. Meals were prepared and served in the metabolic unit located in the Food and Nutrition Building at the University of Nebraska. Subjects maintained their usual schedules as students or employees of the university.

Diet: During the depletion period, nitrogen intake per subject per day averaged 2.15 g, 1.5 g of which was provided by white degerminated corn and the remain-

TABLE 2

Summary of average daily intake, feed required per gram of gain, digestible energy, digestible dry matter and digestible nitrogen (rat study)

Ration [1]	Avg daily feed intake	Feed/gain	Digestible energy	Digestible dry matter	Digestible nitrogen
	g	*g/g*	*%*	*%*	*%*
1	9.09(6.25–11.39) [2]	6.84	85.4	85.3	79.8
2	9.05(6.05–12.53)	7.74	84.8	85.2	75.0
3	12.08(7.86–13.84)	5.19	84.7	85.5	84.2
4	12.58(6.47–16.92)	5.69	83.4	84.3	80.3
5	13.35(6.25–16.08)	4.93	82.7	83.2	85.1
6	13.24(11.00–15.00)	4.51	84.9	84.7	84.3

[1] Ration 1, control corn; 2, control corn plus zein; 3, frost-damaged corn (dough stage); 4, frost-damaged corn (dough stage) plus zein; 5, frost-damaged corn (milk stage); 6, artificially defoliated corn (milk stage). Blister stage occurs about 10 days after fertilization of the ovary, and kernels consist of corn endosperm that has a watery-blistered appearance. Milk stage occurs about 20 days after fertilization of the ovary, and endosperm has a milky appearance. Dough stage occurs about 30 days after fertilization of the ovary, and endosperm has a gelatinous appearance.

[2] Values in parentheses indicate range.

TABLE 3

Amino acid content of control corn and corn defoliated at milk or blister stage [1]

Corn	Nitrogen	Threonine	Isoleucine	Leucine	Lysine	Methionine	Phenyl-alanine	Valine
	%	*mg amino acid/g of corn*						
Control	1.52	3.31	4.03	13.24	3.46	1.81	5.47	5.18
Milk	1.81	3.92	4.97	15.43	3.44	2.35	6.00	6.15
Blister	2.17	4.77	5.18	19.16	4.62	2.48	7.16	7.38
		mg amino acid/g of nitrogen						
Control	1.52	218	265	871	228	119	360	341
Milk	1.81	217	274	852	190	130	331	340
Blister	2.17	220	268	883	213	114	330	340

[1] Values based on 2 separate microbiological analyses of each amino acid. Four levels of sample were used in each microbiological assay. All values were within 5% of the mean values. See footnote in table 2 for explanation of the milk and blister stages of corn.

TABLE 4

Age, height, weight, energy intake and nationality of subjects

Subject	Sex	Age	Body height	Body wt	Daily energy intake	Nationality
		years	*cm*	*kg*	*kcal/kg/day*	
L	M	42	180	73	45	American
M	M	41	170	66	45	Chinese
N	M	29	173	67	48	Korean
O	M	25	170	63	54	Chinese
P	M	33	173	63	49	Korean
R	F	34	160	67	40	Chinese

der by low nitrogen fruits and vegetables. The purpose of this low nitrogen diet was to speed attainment of nitrogen equilibrium with the experimental diet (6).

The depletion period was followed by a 7-day adjustment period in which the male subjects were fed 6 g nitrogen from corn and the female subject, 4.5 g. The difficulty of ingesting 6 g of nitrogen from corn necessitated lowering the level of corn nitrogen for the female subject. During this period subjects adjusted gradually to this level of nitrogen which was then maintained constant throughout the remainder of the study.

Dietary protein during experimental periods was derived from 3 types of corn from the same genetic stock grown in 1963 (single-cross hybrid $B_{14} \times N_6$): control corn, corn defoliated 100% at the milk stage, and corn defoliated 100% at the blister stage of development. The amount of corn consumed by these subjects is shown in table 5. All corn was fed in the ground form. For breakfast it was prepared as mush; for the noon meal, as corn bread; and for the evening meal, as corn and tomato casserole. Butter was added to the corn to make it more palatable. Low protein fruits and vegetables were used to make the diet more acceptable. Calories were adjusted to maintain body weight of individual subjects by appropriate additions of carbonated beverage, sugar, candy and wafers made from sugar, fat and starch. The diet provided 20% calories from fat and was calculated to be adequate in minerals and vitamins.

Analysis: The nitrogen content of the corn, the basal diet, 24-hour urine samples and 5-day fecal composites were analyzed by a modification of the Kjeldahl method (7). Creatinine content of daily urine samples was determined (8) as a measure of the completeness of the urine collections.

RESULTS

Individual and mean nitrogen balances for the 6 subjects in response to the 3 types of corn are shown in table 6. Mean nitrogen balances were: −0.71, −0.55 and −0.19 g/day in response to diets con-

TABLE 5

Composition of diet providing 6 g of nitrogen from various types of corn (human study)

	Amount [1]
	g/day
Cornmeal	395 [2] 332 [3] 277 [4]
Orange juice	80
Peaches	50
Applesauce	100
Pears	100
Tomato juice	100
Jelly	30
Butter	varied [5]
Extra-calorie wafers, sugar, candy, carbonated beverage	varied [6]
Mineral mix [7]	
Vitamin tablet [8]	

[1] 4.5 g of N from corn for subject R.
[2] Control corn.
[3] Defoliated corn (milk stage).
[4] Defoliated corn (blister stage).
[5] Varied with subject in order to provide 20% of total calories from fat.
[6] Amount varied as required to provide isocaloric diets at different levels of cornmeal intake as determined for each subject for weight maintenance. The extra-calorie wafers were made from cornstach, sugar, butter and salt.
[7] Salt mixture manufactured by Nutritional Biochemicals Corporation, Cleveland; 4.3 g salt mixture for each subject provided Ca, 648; P, 0.358; Mg, 0.239; Fe, 0.018; Cu, 0.0024; I, 0.00018; Mn, 0.0024; Zn, 0.00108. Additional Ca and P was provided by a commercial baking powder.
[8] Multiple vitamin tablets manufactured by Division of Miles Laboratories, Inc., Elkhart, Indiana. Each tablet provided: vitamin A, 5000 USP units; vitamin D, 500 USP units; vitamin B_1, 2 mg; vitamin B_2, 2.5 mg; vitamin C, 50 mg; vitamin B_6, 1 mg; vitamin B_{12}, 1 μg; niacinamide, 20 mg; and pantothenic acid, 1 mg.

TABLE 6

Nitrogen balances of 6 adult subjects fed diets containing control corn or corn defoliated at milk stage or blister stage

Subject	Control corn				Milk stage corn				Blister stage corn			
	N intake	N excretion		N balance	N intake	N excretion		N balance	N intake	N excretion		N balance
		Urine	Feces			Urine	Feces			Urine	Feces	
	g/day				*g/day*				*g/day*			
L	6.67	6.43	1.19	−0.95	6.53	5.00	1.56	−0.03	6.68	5.66	1.56	−0.54
M	6.64	4.05	2.39	0.20	6.64	4.13	2.02	0.49	6.69	4.47	2.26	−0.04
N	6.54	5.41	1.02	0.11	6.61	5.12	1.49	0.00	6.59	5.62	1.53	−0.56
O	6.51	4.52	2.19	−0.20	6.59	4.75	2.18	−0.34	6.58	4.84	1.95	−0.21
P	6.49	8.81	0.55	−2.87	6.58	6.82	0.98	−1.22	6.58	7.14	0.97	−1.53
R	4.98	3.75	1.76	−0.53	5.04	3.15	1.95	−0.06	5.09	3.41	2.08	−0.40
Mean	6.30	5.49	1.52	−0.71	6.33	4.82	1.70	−0.19	6.37	5.19	1.73	−0.55

taining control corn, blister stage corn and milk stage corn, respectively. Subject P was in marked negative nitrogen balance with all of the diets. Between-subject variation in each treatment was great. Nitrogen retention tended to be higher in response to corn defoliated at the milk stage than to control corn ($P < 0.07$).

Of the 6 subjects, 4 showed a similar pattern of urinary nitrogen excretion (table 6). These 4 subjects attained the lowest level of urinary nitrogen excretion when the milk-stage corn was fed. The other 2 subjects (O, M) maintained a constant level of urinary nitrogen with the 2 experimental diets. Subject R, the female subject, was fed a diet of lower nitrogen content and hence had a lower urinary nitrogen excretion than the male subjects.

DISCUSSION

The nitrogen content of corn is increased by hail or frost damgae. This damage may be simulated by defoliating the corn plant at the blister or at the milk stage of development, producing a corn of similar nitrogen content. These modifications increase the protein content between 2 and 4%. That the increase in protein content of damaged or defoliated corn is not caused by an increase in the zein fraction of the corn, was demonstrated clearly. The growth of rats fed high-nitrogen corn produced by either of these methods was much superior to that of rats fed regular (not damaged, not defoliated) corn supplemented with zein to provide the same level of nitrogen.

Other workers have observed the effect of naturally produced high-nitrogen corn in which a substantial part of the nitrogen increase was attributed to zein. Hogan et al. (2) observed some improvement in growth but a lower protein efficiency in a rat growth study comparing high-nitrogen with low-nitrogen corn. Hansen and coworkers (9) reported that when the nitrogen content of corn is increased, both zein and zein-free fractions increase, but that the increase in zein is more rapid than the increase in zein-free fractions. In the present study all of the protein added was from zein, whereas in naturally produced high-nitrogen corns zein is proportionally

higher but does not account for the total increase in protein content; therefore the difference in results might be anticipated.

Apparently high-nitrogen corn produced by damage to the plant is different from high-nitrogen corn produced by other methods. Thus Flynn and others (1) reported an increase in the absolute quantities of tryptophan, lysine and methionine but a decrease in the percentage of these amino acids in high-nitrogen as compared with low-nitrogen types of corn. In the present study, the increase in the content of 7 essential amino acids was proportional to the total increase in nitrogen content when high- and low-nitrogen types were compared. Amino acid concentration per gram of nitrogen yielded almost identical values for high- and low-nitrogen types of corn. A logical explanation of the difference between damaged and other high-nitrogen corn is that the nitrogen increase in the damaged corn is relative rather than absolute and results from a decrease in the carbohydrate content of the corn. This would follow if carbohydrate synthesis were impaired in the defoliation of the plant; hail or frost damage might also arrest carbohydrate synthesis. In this event, the protein composition of damaged corn would not differ from that of the undamaged corn. In other types of high-nitrogen corn, the increase in nitrogen is absolute and some proteins in the corn increase more than others.

Results of the nitrogen balance experiment with adult human subjects indicated that the low-nitrogen corn compared favorably with high-nitrogen corn produced by artificial defoliation when nitrogen intakes from the corn were maintained constant. The obvious advantage of the damaged corn is the substantial reduction in quantity of corn needed to provide the same amount of nitrogen and amino acids. Daily amounts of corn required to provide 6 g nitrogen were 395 g (control), 331 g (milk-stage) and 276 g (blister). Lack of statistical significance between responses of subjects to diets providing the various types of corn suggests that these types are approximately equal in nutritional quality, and tends to support the suggestion that the protein composition of the experimental types of corn is the same as that of the control corn. However, there is some indication that corn having its development arrested at the milk stage may have been somewhat superior to the other types of corn tested. The tendency to higher nitrogen balance and lower urinary nitrogen observed in subjects fed this diet cannot be explained in terms of the amino acid composition of the corn. Further studies are indicated to test whether a true superiority exists and to identify other compositional differences among the types of corn which may point to nutrient interrelationships worthy of study.

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Effect of Vitamin D on ^{65}Zn Absorption, Distribution and Turnover in Rats [1]

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ABSTRACT Experiments were designed to study the effect of dietary vitamin D on the absorption, distribution and turnover of ^{65}Zn in growing rats. In the first experiment, whole-animal retention of oral ^{65}Zn by rats fed zinc-supplemented, but not zinc-deficient, diets was slightly ($0.1 > P > 0.05$) increased by continuous supplementation with vitamin D. Retention of injected ^{65}Zn was not similarly enhanced, suggesting a possible effect on ^{65}Zn absorption. In the second experiment ^{65}Zn absorption and turnover were determined by more detailed analysis of whole-animal ^{65}Zn retention patterns, and the distribution of oral and injected ^{65}Zn at 60 hours postadministration was examined. The effect of vitamin D on uptake of oral ^{65}Zn was most pronounced when the vitamin was suddenly added to the diet of previously vitamin D-deficient rats. At 60 hours skeletal, but not soft tissue, uptake of either oral or injected ^{65}Zn was significantly increased by the supplementation of vitamin D to vitamin D-deficient rats. However, skeletal specific activity (% ^{65}Zn per g) was increased by vitamin D only with the orally administered ^{65}Zn. An explanation consistent with all data is that the increased absorption of dietary zinc attributed to vitamin D probably results, not from a direct effect of the vitamin, but from a homeostatic response to the increased need for zinc which accompanies enhanced skeletal growth and calcification.

Zinc absorption and metabolism are affected by a number of dietary variables (1). The antagonistic effect of calcium on zinc utilization noted particularly in swine has been shown in the rat with both practical (2) and semipurified (3) diets to be manifest primarily at the site of zinc absorption. Effects of dietary phosphorus on zinc nutrition have also been studied (1, 3, 4).

The pronounced effect of calcium on zinc nutrition and the role of vitamin D in calcium absorption and metabolism suggest a possible effect of dietary vitamin D on zinc utilization. Published results on such an effect, however, are not in agreement. Whiting and Bezeau (5), through use of conventional balance studies, were unable to show any effect of vitamin D on zinc metabolism in swine when the ration contained 140 ppm zinc, but vitamin D was reported to decrease zinc absorption from a practical ration containing no supplemental zinc. They suggested a possible precipitation of zinc deficiency owing to extensive use of vitamin D supplements. Martin and Patrick (6) reported that addition of vitamin D to the diet of chicks dosed orally with ^{65}Zn increased the amount of ^{65}Zn per gram of tibia when 1% calcium was fed, but produced the opposite effect when the calcium level was raised to 1.6%. A positive effect of vitamin D on zinc metabolism in chickens was indicated further by the work of Kienholz et al. (7) and of Worker and Migicovsky (8). The latter workers fed a single oral dose of vitamin D_3 to rachitic chicks and 36 hours later administered ^{65}Zn either orally or by injection. Chicks dosed orally with vitamin D and later with ^{65}Zn had significantly more ^{65}Zn in the tibiae at 24 hours postadministration than did vitamin D-depleted chicks not dosed with vitamin D. Because the same response was not apparent when ^{65}Zn was injected subcutaneously, it was concluded that vitamin D increased the absorption of zinc from the intestinal tract of the chicken and that vitamin D promoted absorption of many cationic elements in addition to calcium. In contrast, Wasserman (9) reported that duodenal absorption of ^{65}Zn in the chick was not enhanced by vitamin D_3 under the same conditions used to demonstrate significant

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increases due to vitamin D_3 in the absorption of calcium and other alkaline earth cations.

Studies on rats in our laboratory have failed to support the idea that vitamin D exerts a direct effect on zinc utilization. The experiments to be reported indicate that any enhancement of ^{65}Zn absorption by rats supplemented with vitamin D is correlated closely with increased skeletal ^{65}Zn uptake and probably results, not from a direct effect of vitamin D, but from a homeostatic response to the increased need for zinc which accompanies stimulated skeletal calcification and growth. A preliminary report of this work has been published.[2]

MATERIALS AND METHODS

Weanling male rats of the Holtzman strain were used in these experiments. They were housed individually in either stainless steel or galvanized metal cages and were given distilled water ad libitum in glass water bottles. The composition of the basal semipurified diets, containing neither zinc nor vitamin D, is shown in table 1. These diets were supplemented as required with zinc as zinc oxide and vitamin D as calciferol. Rations were assayed for zinc content by atomic absorption spectrophotometry [3] after dry-ashing for 24 hours at 550°.

TABLE 1

Composition of basal diet

	Exp. 1	Exp. 2
	g/kg	*g/kg*
Acid-hydrolyzed casein [1]	180.0	
Alcohol-extracted casein [2]		200.0
Glucose monohydrate [3]	712.7	632.7
Cellulose [4]		60.0
Corn oil	55.0	55.0
Tryptophan	2.2	2.2
Mineral mixture [5]	46.2	46.2
Vitamin mixture [6]	3.9	3.9

[1] Salt-free, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Laboratory-prepared from crude casein.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Solka Floc, Brown Company, New York.
[5] Provided in g/kg of diet: $CaCO_3$, 12.1; $CaHPO_4$, 0.27; KH_2PO_4, 17.06; NaCl, 12.5; $MgSO_4$, 2.43; $Fe(C_6H_5O_7)\cdot 6H_2O$, 0.31; $MnSO_4\cdot H_2O$, 0.058; $CuSO_4$, 0.058; KI, 0.00025; and $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.000175.
[6] Provided in mg/kg of diet; thiamine·HCl, 5; riboflavin, 5; niacin, 25; Ca pantothenate, 20; pyridoxine·HCl, 2.5; folic acid, 0.2; menadione, 0.5; biotin, 0.1; vitamin B_{12}, 0.02; ascorbic acid, 50; inositol, 100; choline chloride, 1400; and (in IU/kg) vitamin A, 5000; α-tocopherol, 110.

High specific activity ^{65}Zn [4] was administered as the zinc-glycine complex at pH 7.4 either orally in 5 g of feed following a 5 to 10 hour fast or by intramuscular injection. To determine the administered dose, a whole-animal gamma-scintillation detector [5] was used to assay each rat for ^{65}Zn content exactly 4 hours after the feed containing ^{65}Zn was first offered or ^{65}Zn was injected (losses were negligible to that point and diet consumption was complete). The rats were assayed for ^{65}Zn at numerous time intervals thereafter to determine whole-animal ^{65}Zn retention. The data, corrected for coincidence loss, background radiation, radioactive decay of the isotope and minor fluctuations in counting efficiency, were expressed as a percentage of the initially administered dose and plotted on a logarithmic scale as a function of time. For experiment 2, the percentage absorption of the orally administered ^{65}Zn and biological half-life of 3 resolved components of the ^{65}Zn retention curves were determined as outlined by Heth and Hoekstra (2). A CDC 1604 computer was programmed to perform all necessary calculations.

Organ and tissue samples obtained by dissection of some of the rats after ^{65}Zn administration in experiment 2 were assayed for ^{65}Zn either in the whole-animal detector or in a well-type crystal scintillation counter.[6] The carcasses which remained after dissection were also assayed for ^{65}Zn and were then fed to a colony of *Dermestes lardarius*, insects which feed on dead animal tissue. The insect colony consumed all of the soft tissue and reduced the rat carcasses to the skeletal systems within 3 to 4 days. The bones were then rinsed in acetone to remove any remaining insects, crushed and assayed for ^{65}Zn in the crystal scintillation detector. By subtracting the ^{65}Zn content of the skeletal system from that of the carcass before it was fed to the *Dermestes* colony, the ^{65}Zn

[2] A preliminary report of this research was presented at the 1965 annual meeting of the American Society of Animal Science at East Lansing, Michigan.
[3] Model 214 atomic absorption spectrophotometer, manufactured by the Perkin-Elmer Company, Norwalk, Connecticut.
[4] The radiozinc was obtained as $^{65}ZnCl_2$ in HCl from Oak Ridge National Laboratory, Oak Ridge, Tennessee.
[5] Armac scintillation detector model 440 manufactured by Packard Instrument Company, Inc., La-Grange, Illinois.
[6] Well-type crystal scintillation detector manufactured by Nuclear-Chicago, Inc., Chicago.

content of the soft tissue was determined. All ^{65}Zn measurements were corrected for coincidence loss, background, radioactive decay and difference in counting efficiency between the 2 instruments. The data were then expressed as a percentage of the total initial ^{65}Zn dose.

All statistical comparisons of treatment means were by Student's *t* test and Duncan's new multiple range test (10). An inverse sine transformation was routinely applied to all percentage data, as suggested by Steel and Torrie (10).

RESULTS

Experiment 1: Effect of vitamin D on ^{65}Zn retention. The purpose of this experiment was to compare the effect of vitamin D deficiency and continuous supplementation of vitamin D on whole-animal retention of oral and injected ^{65}Zn by rats receiving either a zinc-deficient or a zinc-supplemented diet. Sixteen rats received the basal diet shown in table 1 with no source of vitamin D, and 16 additional rats received the same diet supplemented with 8000 IU vitamin D_2/kg. Within each level of dietary vitamin D, 8 rats were fed the zinc-deficient (2.0 ppm) diet and the remaining 8 received the zinc-supplemented (20.7 ppm) diet. Rats were pair-fed within a given level of dietary zinc to eliminate any differences in size or growth rate from consideration of the effects of vitamin D on ^{65}Zn retention.

After 3 weeks on experiment, 1 μCi ^{65}Zn was administered by diet after a 5-hour fast to 4 of the rats on each dietary regimen and by intramuscular injection to the remaining 4 rats. Whole-animal ^{65}Zn measurements were made as described in the Methods section at 12 to 30-hour intervals until 170 hours (injected ^{65}Zn) or 260 hours (oral ^{65}Zn) postadministration.

Whole-animal retention of the ^{65}Zn is plotted as a function of time for the oral dose in figure 1 and for the injected dose in figure 2. The extremely tenacious retention of ^{65}Zn by zinc-deficient rats has been pointed out previously (3); this effect was noted whether the ^{65}Zn was fed or injected, indicating that zinc-deficient rats exhibit both a remarkably high absorption and a very slow rate of excretion of body zinc.

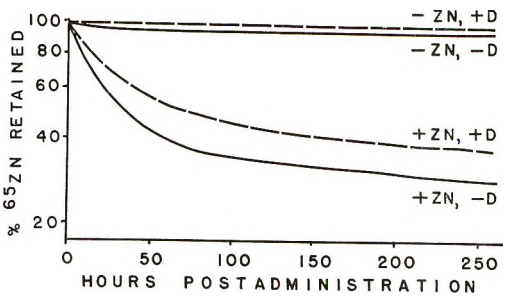

Fig. 1 Exp. 1. Effect of dietary zinc and vitamin D on whole-body retention of ^{65}Zn-glycine administered by diet. Four rats/treatment group were fed the respective diets for 3 weeks prior to and 11 days after a single ^{65}Zn administration. The glucose-casein hydrolysate diets contained: −Zn, 2 ppm Zn; +Zn, 21 ppm; −D, no vitamin D added to diet; +D, 8,000 IU/kg. Rats were pair-fed within each level of dietary zinc but not between zinc levels.

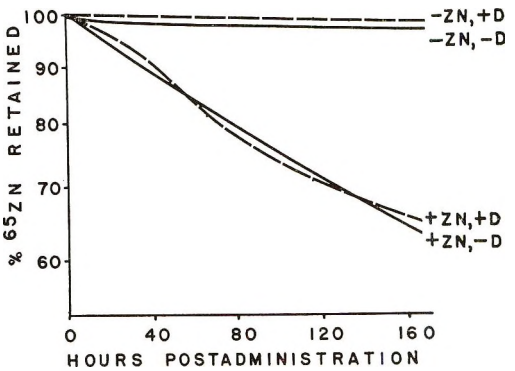

Fig. 2 Exp. 1. Effect of dietary zinc and vitamin D on whole-body retention of ^{65}Zn-glycine injected intramuscularly. Conditions were as described for figure 1.

The presence of vitamin D resulted in a slightly increased retention of oral ^{65}Zn at both levels of dietary zinc. This effect was not statistically significant in either case, although it approached significance ($P < 0.1$) for the adequate zinc diet. Since the small difference in ^{65}Zn retention between the 2 levels of dietary vitamin D appeared by 50 hours postadministration and remained essentially constant thereafter, it was probably the result of a difference in the amount of the original dose initially absorbed. This conclusion is supported by the lack of effect of vitamin D on the rate of turnover of injected ^{65}Zn (fig. 2) at either level of dietary zinc. This experi-

ment has been repeated both with and without pair-feeding; in each case, a slight increase in retention of the orally administered ^{65}Zn dose by the vitamin D- supplemented rats was noted, but in no case was the difference statistically significant.

Experiment 2: Effect of vitamin D supplementation on the absorption, distribution and turnover of ^{65}Zn. This experiment was designed to study the effects of sudden supplementation of vitamin D to vitamin D-deficient rats and to obtain data for the rat more directly comparable to those reported for chicks by Worker and Migicovsky (8). Studies on vitamin A were included for comparison to the effects of vitamin D.

One hundred and sixty weanling rats were assigned at random to 5 treatment groups of 32 animals each. The composition of the control diet fed to treatment group 5 is that shown in table 1, but it contained in addition 10,000 IU vitamin D_2/kg by calculation and 26.3 ppm zinc by analysis. For the other treatment groups, vitamin D was omitted in treatments 1 and 2 and vitamin A was omitted in treatments 3 and 4. Feed was supplied ad libitum.

After the animals had been fed the experimental diets for 21 days, rats in treatment 2 (−D) received 2000 IU vitamin D_2 and those in treatment 4 (−A) received 1000 IU vitamin A. In each case the vitamin was administered orally in 5 g of feed following a 10-hour fast. Thereafter, treatment groups 2 and 4 were fed the nondeficient control diet ad libitum. Rats in treatment groups 1 and 3 were fed the original deficient diets (−D and −A, respectively) thoughout the experiment. Thirty-six hours after vitamin supplementation to groups 2 and 4, 1 μCi of ^{65}Zn was administered to each animal on experiment. Of the 32 rats in each treatment group, 20 received the ^{65}Zn orally in 5 g of feed following a 10-hour fast and the remaining 12 rats received the ^{65}Zn by intramuscular injection. More rats were given the isotope orally than by injection because of the greater variation which is encountered in retention of ^{65}Zn administered by the oral route. The initial administered dose was measured as described in the Methods section.

All rats were assayed for ^{65}Zn content 5 times during the 60 hours after the initial measurement. At 60 hours, 10 of the 20 rats in each group which had received ^{65}Zn orally and 6 of the 12 that had received ^{65}Zn by injection were killed and dissected. The liver, kidneys, pelt, gastrointestinal tract and contents and remaining carcasses were weighed and assayed for ^{65}Zn content, after which the carcasses were fed to the *Dermestes* colony to obtain the skeletal systems, which were similarly weighed and analyzed for ^{65}Zn. Rats not killed at 60 hours were assayed for ^{65}Zn at 18 separate time-intervals between 60 and 612 hours postadministration.

As shown in figure 3, the response to vitamin supplementation was rapid; within several days the rats given the supplements (treatment groups 2 and 4) weighed almost as much as the controls (group 5) and were growing at a significantly faster rate than the deficient animals (groups 1 and 3). The vitamin D-deficient rats continued to grow slowly throughout the course of the experiment, but those deficient in vitamin A reached a growth plateau and began losing weight at about 4 weeks with the experimental diets. Most of the rats in treatment group 3 died of vitamin A deficiency before 612 hours postadministration of ^{65}Zn.

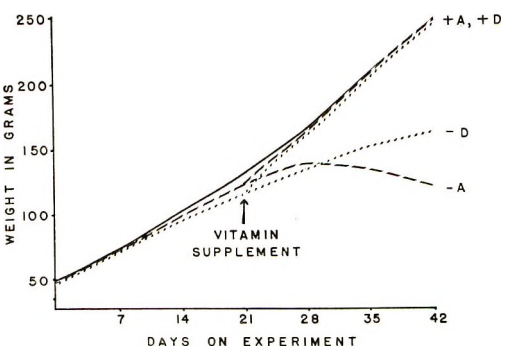

Fig. 3 Exp. 2. Growth of rats (5 treatment groups of 32 rats each) fed a glucose-casein diet with and without vitamins A and D. Treatments were: −A, vitamin A omitted from diet throughout experiment; −D, vitamin D omitted from diet throughout experiment; +A+D, corresponding groups with respective deficiency corrected at 21 days and a group fed vitamin A (5,000 IU/kg diet) and vitamin D (10,000 IU/kg diet) throughout experiment.

Data obtained by resolving the whole-animal ^{65}Zn retention curves and determining the percentage of ^{65}Zn absorption as described previously (2) are shown in table 2. Mean absorption of dietary ^{65}Zn was lower in this experiment than values reported previously for a similar semipurified diet (3); this difference was shown to result primarily from the replacement in the diet of casein hydrolysate with alcohol-extracted casein and to the inclusion of 6% cellulose [7] as a source of dietary bulk. Within the present experiment, however, none of the treatment means for ^{65}Zn absorption differed significantly from the others. The slightly increased absorption attributed in experiment 1 to continual supplementation with vitamin D was not noted in the corresponding comparison in the present data (treatment groups 5 vs. 1). However, rats in treatment groups 3 and 4, which received vitamin D continuously in the diet but were vitamin A-deficient for all or part of the experiment did absorb somewhat more ^{65}Zn than the vitamin D-deficient rats in group 1. In this respect, the absorption data for treatments 3 and 4 may be more representative of the type of response usually observed in a comparison of long-term vitamin D supplementation and deficiency than the corresponding value for treatment 5. The vitamin D-deficient rats in group 2, which had been given the vitamin D supplement 36 hours before ^{65}Zn administration, absorbed somewhat more ^{65}Zn than the vitamin D-deficient rats in group 1 that received no supplement. However, like the effect of continuous vitamin D supplementation observed in experiment 1, this difference only approached significance ($P < 0.1$).

Supplementation of vitamin A to rats deficient in that vitamin did not enhance ^{65}Zn absorption. If anything, the effect of the vitamin A was to lower ^{65}Zn absorption, although the difference was not significant ($P > 0.1$).

Table 2 also shows the mean biological half-life for the first components of the resolved ^{65}Zn retention curves; these values reflect the rate of passage of ingested ^{65}Zn through the gastrointestinal tract. None of these means was significantly different from the others. The biological half-life of 2 further segments of the ^{65}Zn retention curves (table 2) represents turnover of absorbed or injected ^{65}Zn. No significant difference in biological half-life of the 108- to 324-hour component was noted between any of the groups for a given route of ^{65}Zn administration. This is in agreement with the results shown in figure 2. For the 372- to 612-hour component, the biological half-life of injected ^{65}Zn was significantly ($P < 0.05$) shorter

[7] Solka Floc, Brown Company, New York.

TABLE 2

Effects of vitamins A and D on the absorption and turnover of ^{65}Zn

Treatment [1]	Method of ^{65}Zn administration	^{65}Zn absorption	Biological half-life of ^{65}Zn		
			First component (0–60 hr)	Second component (108–324 hr)	Third component (372–612 hr)
		%	*hours*	*hours*	*hours*
1 (−D)	oral	13.4 ± 1.2 [2]	12.5 ± 0.9 [2]	435 ± 23 [2]	826 ± 44 [2]
	injection			502 ± 16	914 ± 36
2 (−D, then +D)	oral	19.8 ± 3.1	11.6 ± 1.0	466 ± 16	1153 ± 42
	injection			453 ± 21	1019 ± 49
3 (−A)	oral	19.0 ± 1.8	10.1 ± 1.0	437 ± 17	undetermined [3]
	injection			541 ± 24	undetermined [3]
4 (−A, then +A)	oral	17.2 ± 1.8	13.5 ± 1.8	447 ± 30	undetermined [3]
	injection			541 ± 24	undetermined [3]
5 (+D, +A)	oral	13.4 ± 2.2	9.9 ± 0.8	480 ± 27	1063 ± 31
	injection			484 ± 26	989 ± 17

[1] (−D) and (+D) indicate without and with vitamin D; (−A) and (+A) indicate without and with vitamin A.

[2] Treatment means ± SE.

[3] Data for 372–612 hour component of whole-animal retention curve for treatments 3 and 4 not determined due to death of most of the vitamin A-deficient animals before 600 hours postadministration.

than that of the absorbed isotope for rats given the vitamin D supplement, but not for vitamin D-deficient rats. In addition, rats that received the vitamin D supplement had a longer ^{65}Zn biological half-life than vitamin D-deficient rats for the oral dose ($P < 0.01$), but for the injected dose, the difference was not significant. These observations suggest that more of the ^{65}Zn absorbed by vitamin D-supplemented rats was turning over less rapidly than that absorbed by vitamin D-deficient rats and may therefore have been in bone.

The distribution of ^{65}Zn in rats at 60 hours postadministration is presented in table 3. Supplementation of vitamin A to rats deficient in that vitamin had no significant effect on the amount of either oral or injected ^{65}Zn present in any of the body components analyzed.

Although the effect of vitamin D on the absorption of ^{65}Zn as determined by analysis of whole-animal retention curves (table 2) was not significant, the data in table 3 indicate that, at 60 hours postadministration, the vitamin D-supplemented

TABLE 3

Effects of vitamins A and D on the distribution of ^{65}Zn at 60 hours postadministration

Sample	Treatment [1]	Portion of total initial ^{65}Zn present in sample at 60 hours postadministration	
		Oral ^{65}Zn	Injected ^{65}Zn
		%	%
Whole animal	1 (−D)	19.2 ±2.8 [2]	83.0 ±0.5 [2]
	2 (−D, then +D)	16.2 ±1.5	78.0 ±2.1
	3 (−A)	16.3 ±1.6	79.4 ±1.2
	4 (−A, then +A)	18.0 ±1.5	81.7 ±1.0
	5 (+D, +A)	17.5 ±1.6	81.0 ±1.3
Gastrointestinal tract and contents	1 (−D)	9.1 ±1.8	12.2 ±0.8
	2 (−D, then +D)	3.7 ±0.5	11.7 ±0.7
	3 (−A)	5.0 ±0.9	10.4 ±0.5
	4 (−A, then +A)	6.3 ±0.9	11.2 ±0.7
	5 (+D, +A)	4.8 ±0.6	9.8 ±0.3
Liver	1 (−D)	1.2 ±0.2	6.5 ±0.3
	2 (−D, then +D)	1.2 ±0.2	5.8 ±0.2
	3 (−A)	1.2 ±0.2	5.7 ±0.2
	4 (−A, then +A)	1.4 ±0.1	6.3 ±0.3
	5 (+D, +A)	1.5 ±0.2	6.6 ±0.2
Kidneys	1 (−D)	0.22±0.02	0.97±0.04
	2 (−D, then +D)	0.22±0.02	0.90±0.05
	3 (−A)	0.25±0.03	1.11±0.04
	4 (−A, then +A)	0.21±0.03	1.15±0.06
	5 (+D, +A)	0.26±0.03	1.20±0.03
Pelt (skin and hair)	1 (−D)	1.6 ±0.2	12.3 ±1.7
	2 (−D, then +D)	1.7 ±0.1	8.4 ±0.4
	3 (−A)	1.8 ±0.2	9.9 ±0.8
	4 (−A, then +A)	1.6 ±0.2	10.3 ±0.6
	5 (+D, +A)	1.9 ±0.3	9.8 ±0.6
Other soft tissues	1 (−D)	4.8 ±0.1	33.4 ±0.7
	2 (−D, then +D)	4.6 ±0.4	27.6 ±0.8
	3 (−A)	4.6 ±0.6	31.6 ±1.3
	4 (−A, then +A)	4.8 ±0.4	32.2 ±1.5
	5 (+D, +A)	5.7 ±0.7	34.9 ±1.1
Skeleton	1 (−D)	1.9 ±0.2	14.8 ±0.7
	2 (−D, then +D)	3.9 ±0.3	19.8 ±0.6
	3 (−A)	3.1 ±0.3	16.6 ±0.6
	4 (−A, then +A)	3.0 ±0.2	16.1 ±0.9
	5 (+D, +A)	3.0 ±0.3	15.7 ±0.4

[1] (−D) and (+D) indicate without and with vitamin D; (−A) and (+A) indicate without and with vitamin A.

[2] Treatment means ± SE.

rats had a significantly smaller fraction of the initial oral ^{65}Zn dose remaining in the digestive tract than the unsupplemented vitamin D-deficient rats of group 1. This effect was greatest ($P < 0.01$) when the vitamin D supplement was suddenly given to previously vitamin D-deficient rats (group 2), but was also significant ($P < 0.05$) for the rats which had been continuously fed vitamin D (groups 3, 4 and 5).

Vitamin D supplementation also resulted in an increased incorporation of oral ^{65}Zn into the skeleton. Again, the effect was most marked ($P < 0.001$) following supplementation of vitamin D to previously deficient rats, but was also noted ($P < 0.01$) in the case of continuous vitamin D supplementation. The extent of increased deposition of oral ^{65}Zn in the skeleton appeared to be correlated with the removal of ^{65}Zn from the digestive tract.

Vitamin D had no significant effect on the amount of oral ^{65}Zn found at 60 hours postingestion in liver, kidneys, pelt or other soft tissues. Also, it was found earlier [8] that vitamin D had no significant effect on soft-tissue zinc concentrations in rats fed the same type of semipurified diet and receiving various levels of dietary zinc.

The effects of vitamin D on zinc metabolism may relate entirely to the stimulation by vitamin D of bone growth and calcification. This effect is readily observed by comparing treatment groups 1 and 2 in table 4. The average weight of the skeletal system (rats given ^{65}Zn orally and by injection combined) was 4.4 g for the vitamin D-supplemented vs. 3.6 g for the vitamin D-deficient rats ($P < 0.01$), indicating that about 20% of the total weight of the skeletal system was deposited by the vitamin D-supplemented rats during the 96 hours from vitamin supplementation to killing. Enhanced skeletal uptake of ^{65}Zn was measured during the final 60 hours of this 96-hour period. In view of the relatively high zinc content of the skeleton, it is probably not unexpected that animals responding to vitamin D supplementation absorbed more of an orally administered dose of ^{65}Zn than did D-deficient rats. Supplementation with vitamin A did not stimulate skeletal growth during the interval studied (5.3 g for treatment group 3 vs. 4.9 g for treatment group 4; $P > 0.1$) and, as noted above, had no significant effect on uptake of dietary ^{65}Zn.

Unlike the results reported for chicks by Worker and Migicovsky (8), an effect ($P < 0.001$) of supplemental vitamin D on skeletal ^{65}Zn was also observed (table 3) when the isotope was injected intramuscularly, although the relative difference was less than in the case of the orally administered ^{65}Zn. The vitamin D-supplemented rats had a correspondingly smaller fraction of the injected dose present in the pelt ($P < 0.05$) and in the soft tissue ($P < 0.001$) than unsupplemented vitamin D-deficient rats. Liver and kidney showed a similar effect to other soft tissues, but the differences were not statistically significant. A likely explanation for the difference between the present results and those reported by Worker and Migicovsky (8) is that in the latter case only 24 hours were allowed between ^{65}Zn injection and killing

[8] Becker, W. M., and W. G. Hoekstra, unpublished data.

TABLE 4

Effects of vitamins A and D on skeletal weight and skeletal ^{65}Zn

Treatment [1]	Oral ^{65}Zn		Intramuscular ^{65}Zn	
	Wt of skeleton [2]	Specific activity [3]	Wt of skeleton [2]	Specific activity [3]
	g	*%/g*	*g*	*%/g*
1 (−D)	3.7 ± 0.2 [4]	0.53 ± 0.05 [4]	3.5 ± 0.2 [4]	4.3 ± 0.2 [4]
2 (−D, then +D)	4.4 ± 0.1	0.89 ± 0.08	4.6 ± 0.2	4.4 ± 0.3
3 (−A)	5.5 ± 0.2	0.57 ± 0.05	4.9 ± 0.2	3.4 ± 0.1
4 (−A, then +A)	4.8 ± 0.2	0.63 ± 0.04	5.0 ± 0.2	3.2 ± 0.1
5 (+D, +A)	4.7 ± 0.1	0.65 ± 0.06	4.8 ± 0.2	3.2 ± 0.1

[1] (−D) and (+D) indicate without and with vitamin D; (−A) and (+A) indicate without and with vitamin A.
[2] Separated from soft tissues by *Dermestes lardarius* (see text).
[3] Percentage of total initial ^{65}Zn/g of skeleton at 60 hours postadministration.
[4] Treatment means ± SE.

and that this was insufficient time for significant skeletal growth compared with 60 hours in the present study. When skeletal ^{65}Zn content was expressed as specific activity (percentage of total initial ^{65}Zn/g skeleton, table 4) to correct for differences in total skeletal size, there was no effect on injected ^{65}Zn of supplementing vitamin D to vitamin D-deficient rats. For the orally administered ^{65}Zn, however, increased skeletal uptake by the vitamin D-supplemented rats far exceeded the difference in skeletal size, such that a large difference ($P < 0.001$) also existed in specific activity of the bone between the vitamin D-deficient and vitamin D-supplemented rats. This is further verification that an increased ^{65}Zn absorption occurred following vitamin D supplementation of vitamin D-deficient rats. When the label was in the small pool of zinc in the gut, an increased absorption would substantially increase the specific activity of the circulating zinc pool supplying zinc for bone growth and calcification. In contrast, when the much larger body zinc pools were labeled (as by injection) such increased absorption of stable zinc would have little effect on the specific activities of the circulating zinc pool and thus of bone zinc. In fact, the increased absorption of stable zinc would have decreased somewhat the specific activity of the circulating zinc pool, such that despite the stimulation of bone growth due to the added vitamin D, no difference was observed in the deposition of ^{65}Zn per unit weight of bone in the presence or absence of vitamin D.

DISCUSSION

The results of these experiments indicate that the uptake of oral ^{65}Zn by rats is enhanced slightly in the presence of adequate vitamin D and that this effect is more noticeable when the vitamin D supplement is suddenly given to rats previously deficient in vitamin D. Even in the latter case, however, there was no significant increase in ^{65}Zn content of soft tissues, and in other experiments, dietary vitamin D, whether deficient, adequate or excessive, had little or no effect in alleviating or aggravating the zinc-deficiency syndrome in rats [9] or swine.[10]

The effect of vitamin D on ^{65}Zn absorption in the experiments reported here was closely correlated with an increased deposition of ^{65}Zn in the skeleton. The role of zinc in bone is not clearly defined, but bone is known to contain relatively high amounts of zinc. Work by Haumont (11) and by Haumont and Vincent (12) suggests that zinc is localized at the boundary between mineralized bone and preosseous substance and is deposited exactly at the time when calcification begins. When supplemental vitamin D stimulates bone calcification and thereby increases the need for zinc in bone, it seems logical that the additional zinc required is drawn from circulating body pools and that these in turn are replenished by an enhanced absorption of dietary zinc from the intestinal tract.

Such an indirect effect of vitamin D on zinc absorption is supported by the observed correlation between skeletal growth, skeletal uptake of ^{65}Zn and absorption of dietary ^{65}Zn. Rats continually supplemented with vitamin D (in exp. 1 and in treatments 3, 4 and 5 of exp. 2) retained slightly more oral ^{65}Zn than vitamin D-deficient rats and had deposited a larger proportion of ^{65}Zn in the skeletal system by 60 hours postingestion. A more pronounced effect of vitamin D on zinc absorption was noted when the vitamin was administered to previously deficient animals (in exp. 2) and was closely correlated with a sudden increase in skeletal growth and a much greater uptake of ^{65}Zn by bone than was observed for either the vitamin D-deficient or continuously supplemented rats.

It appears reasonable, therefore, to conclude that the increase in uptake of ^{65}Zn by skeletal tissue when vitamin D-deficient animals are treated with vitamin D is due to stimulation of bone growth and calcification by the vitamin and that the increased absorption of dietary zinc attributed to the vitamin is a homeostatic response to the increased need for zinc which results. Such an explanation would not be in disagreement with the results of Worker and Migicovsky (8) nor with the results of Wasserman (9). Moreover,

[9] See footnote 8.

[10] Roberts, H. F., W. G. Hoekstra and R. H. Grummer 1961 Vitamin D, zinc and calcium in swine nutrition. J. Animal Sci., 20: 950 (abstract).

Cotzias et al. (13) have demonstrated that a homeostatic mechanism controlling zinc absorption does indeed exist.

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Effect of Altering the Dietary Cation-Anion Ratio on Food Consumption and Growth of Young Chicks

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ABSTRACT Eight-day-old chicks were used in a series of 3 experiments to study the effects of adding acid, base or a combination of these to an amino acid-cornstarch diet. The cation-anion ratio of the diet was reduced from 1.2 to 0.6 by adding increasing levels of L-glutamic acid hydrochloride and was increased from 1.2 to 2.4 with sodium or potassium carbonates or a combination of the two. Food consumption and weight gain were maximized when the ratio was in the range of 1.2 to 1.8. A ratio of 0.6 nearly completely inhibited growth. Sodium and potassium chlorides did not reduce food consumption or weight gain when added to the basal diet in equivalents equal to the highest level of hydrochloride. Excess calcium did not alleviate the growth depression due to excess chloride, whereas magnesium partially overcame the depression. The response of chicks to diets with ratios of greater than 1.8 depended upon the cation added. A combination of sodium and potassium was not detrimental but either cation alone reduced weight gain. It was found that the chick possesses kidney glutaminase I activity, but the activity of the enzyme did not increase when dietary acidity was increased.

The growth response of chicks consuming diets containing essential minerals at optimal or superoptimal levels of requirements has been demonstrated to vary widely with different experimental conditions. Leach et al. (1) have reported that excess dietary sulfate depressed the growth of chicks. Nesheim et al. (2) likewise have reported that excess chloride was deleterious to the chick. The latter workers reported no depression in growth when sodium and potassium were added to the diets in amounts to equal the equivalents of added anion. Correll (3) and Scott et al. (4) have reported that excess dietary sodium was toxic to chicks when the sodium was added to the diet as the salt of a metabolizable anion. The present study was an attempt to define conditions in which chloride, sodium and potassium cause a depression in the growth of young chicks.

METHODS

Eight-day-old chicks, from the mating of New Hampshire males to Columbian females, were allotted to treatments in a manner to assure uniformity of initial weight. Mean initial weights were 76, 77 and 76 g for experiments 1, 2 and 3, respectively. Male chicks were used in experiments 1 and 3, females in experiment 2. The sequence of feeding and fasting prior to and at the termination of each experiment was identical to that described by Dean and Scott (5). Duplicate groups of 10 chicks each per treatment were housed in electrically heated brooders and received the experimental diets for a period of 6 days. Tap water was provided ad libitum. Individual initial and final weights and replicate food consumption were measured.

The composition of the basal diet is shown in table 1. The anion content of the diet was increased by substituting L-glutamic acid hydrochloride for L-glutamic acid on an equal molar basis (treatments 2–6) in the amino acid mixture. Calcium chloride dihydrate, magnesium chloride hexahydrate and sodium chloride plus potassium chloride (treatments 7, 8 and 9, respectively) were added to the diet in quantities to equal the chloride provided by the highest level of glutamic acid hydrochloride (treatment 6). The cation content of the diet was increased by adding sodium carbonate and potassium carbo-

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TABLE 1

Composition of basal chick diet

	%
Cornstarch	55.96
Amino acid mixture [1]	19.48
Corn oil	15.00
Mineral mixture [2]	5.36
Cellulose [3]	3.00
$NaHCO_3$	1.00
Choline chloride	0.20
Vitamin mixture [4]	+
α-Tocopheryl acetate [5]	+
Antioxidant [6]	+

[1] Zimmerman and Scott (7), mixture B; provided the following per 100 g of diet: (in grams) L-arginine·HCl, 1.21; L-histidine·HCl·H_2O, 0.41; L-lysine·HCl, 1.19; L-tyrosine, 0.45; L-tryptophan, 0.15; L-phenylalanine, 0.50; DL-methionine, 0.35; L-cystine, 0.35; L-threonine, 0.65; L-leucine, 1.20; L-isoleucine, 0.80; L-valine, 0.82; glycine, 1.20; L-proline, 0.20; and L-glutamic acid, 10.00.

[2] Zimmerman and Scott (7); provided the following per 100 g of diet: (in grams) $Ca_3(PO_4)_2$, 2.80; $CaCO_3$, 0.30; K_2HPO_4, 0.90; NaCl, 0.88; $MgSO_4·7H_2O$, 0.35; $MnSO_4·H_2O$, 0.065; ferric citrate, 0.050; $ZnCO_3$, 0.010; KI, 0.004; $CuSO_4·5H_2O$, 0.002; H_3BO_3, 0.0009; Na_2MoO_4, 0.0009; and $CoSO_4·7H_2O$, 0.0001.

[3] Solka Floc, Brown Company, Boston, Massachusetts.

[4] Klain et al. (8), less α-tocopheryl acetate.

[5] 20 mg/kg diet.

[6] Santoquin, 125 mg/kg diet; Monsanto Company, St. Louis.

nate, either alone (treatments 10–15, 16–21) or in an equimolar combination (treatments 22–27) to the basal diet. All mineral additions replaced equal quantities of starch.

The relative levels of dietary inorganic acids and bases may be expressed as the cation-anion ratio. The number of physiological milliequivalents of calcium magnesium, sodium and potassium constitute the cation and phosphate, chloride and sulfate constitute the anion. The assumptions made in the calculation were (a) 75% of the dietary calcium was absorbed resulting in either retention or urinary excretion and that amount apparently unabsorbed was of no physiological importance (i.e., 1.5 physiological equivalents/mole of calcium); (b) 100% of the dietary phosphorus was physiologically important and was equal to 1.75 equivalents of anion/mole at pH 7.4; and (c) 100% of the dietary magnesium, sodium, potassium, chloride and sulfate were physiologically important (i.e., magnesium and sulfate contributed 2 equivalents/mole, and sodium, potassium and chloride one equivalent/mole). The calculated cation-anion ratio of the basal diet used in these experiments was approximately 1.2

$$\left(\frac{85 \text{ mEq cation}/100 \text{ g diet}}{70 \text{ mEq anion}/100 \text{ g diet}}\right).$$

At the termination of experiment 1, two chicks from each replicate were decapitated, the kidneys were removed, frozen immediately on dry ice and stored in a freezer maintained at − 15°. Kidney glutaminase I activity was determined by a modification of the procedure described by Goldstein (6). A kidney was homogenized in 19 volumes of ice-cold 0.9% saline with a Potter-Elvehjem homogenizer. Two-tenths milliliter of homogenate was incubated for 20 minutes at 37° with 0.5 ml of 0.05 M Tris buffer pH 8.0, 0.2 ml of 0.5 M phosphate buffer pH 8.0 and 0.1 ml of 0.1 M L-glutamine. The reaction

TABLE 2

Effects of hydrochloride and chloride salts on food consumption and growth

Exp. no.	Treatment no.	Dietary additive	Chloride [1] added	Cation [2] / anion	Diet consumed/ chick/day	Gain/day
			%		*g*	*g*
1,2,3	1	none [3]	—	1.2	18.0	11.5 ± 0.2 [4]
1	2	HCl [5]	0.28	1.1	16.5	10.6 ± 0.3
1	3	HCl	0.55	1.0	16.3	9.7 ± 0.2
1	4	HCl	0.89	0.9	14.4	7.0 ± 0.3
1	5	HCl	1.31	0.8	11.9	5.2 ± 0.3
1,3	6	HCl	2.41	0.6	6.8	1.1 ± 0.2
1	7	$CaCl_2·2H_2O$	2.41		8.3	2.1 ± 0.3
3	8	$MgCl_2·6H_2O$	2.41		13.2	6.3 ± 0.2
3	9	NaCl + KCl [6]	2.41		16.9	10.9 ± 0.4

[1] Basal diet contained 1.047% chloride.

[2] Milliequivalents of physiological cation:mEq physiological anion/unit of diet.

[3] Basal diet.

[4] Mean ± SE of 20 chicks/treatment/experiment.

[5] Added as L-glutamic acid hydrochloride and replaced equal moles of L-glutamic acid in the amino acid mixture.

[6] Sodium chloride and potassium chloride added in equal molar quantities.

was stopped by the addition of 0.2 ml of 30% trichloroacetic acid to the incubation mixture. Ammonia was measured by microdiffusion and nesslerization. Glutamine and homogenate blanks were included in each series of samples.

RESULTS

Effects of excess anions. The food consumption and growth data are presented in table 2. As the average daily gains of the chicks receiving the basal diet (treatment 1) were not significantly different between experiments, the data from the 3 experiments were combined. Food consumption and weight gain were depressed when the chloride content of the diet was increased without increasing the cation content (treatments 2–6). A cation-anion ratio of 1.0 (treatment 3) depressed weight gain compared with the basal. As the level of hydrochloride was increased, food consumption and weight gain were depressed to the point where the chloride of diet 6 nearly completely inhibited growth. Diets 7, 8 and 9 contained chloride equal to diet 6. Chloride per se was not detrimental to the chick when fed as the sodium and potassium salts (treatment 9). Calcium chloride (treatment 7) depressed weight gain and food consumption to nearly the same extent as hydrochloride. This indicates either a preferential absorption of chloride or absorption of both and different routes of excretion, namely, calcium via intestine and chloride via kidney. Magnesium chloride (treatment 8) depressed weight gain and food consumption less than an equal number of equivalents of hydrochloride (treatment 6). Both criteria for treatment 8 were nearly equal to those for treatment 4 with a dietary cation-anion ratio of 0.9. The percentage of the added magnesium which served as a physiologically active cation was calculated. The anion content of the diet was known and the weight gains of chicks receiving the hydrochloride diets were used as a standard curve. The cation-anion ratio of the magnesium chloride treatment was then extrapolated from the standard curve. The difference between the calculated cation content of diets 8 and 1 (basal diet) was the milliequivalents of magnesium which served as a physiological cation. The portion of the added magnesium which was physiologically active was calculated to be 50%. A similar percentage of excess magnesium excretion via the kidneys of rats receiving magnesium chloride has been observed in our laboratory (unpublished data).

Glutaminase I activity was detected in chick kidneys. The specific activities are shown in table 3. There was no significant trend toward increased activity with increased acidity of the diet. This is in contrast with the rat (9, 10) and sheep (unpublished data). The role of kidney glutaminase enzymes in animals is the formation of ammonium ion for association with excreted excess anions when insufficient amounts of inorganic cations are provided in the diet. The physiological importance of chick kidney glutaminase remains to be established.

Effects of excess cations. The results of adding sodium, potassium or a combination of the two, as the carbonates, to the basal diet of the chick are shown in table 4. The highest levels of added sodium (treatments 14 and 15) reduced food consumption and weight gain. Excess sodium appeared to be slightly toxic as evidenced by the death of a total of 5 chicks in treatments 13–15. On an equal equivalents basis, excess potassium tended to depress weight gains more than sodium. Although potassium-supplemented diets appeared to support a slower rate of gain than sodium, the carcass dry matter gain may not have been different. The chicks receiving the excessive levels of sodium exhibited edema, which may have been

TABLE 3

Kidney glutaminase I activity of chicks consuming diets varied in chloride content

Treatment [1] no.	Dietary additive	Cation / anion	Specific activity [2]
1	none [3]	1.2	0.97 ± 0.08 [4]
2	HCl [5]	1.1	0.73 ± 0.13
3	HCl	1.0	0.98 ± 0.13
4	HCl	0.9	0.87 ± 0.12
5	HCl	0.8	1.51 ± 0.13
6	HCl	0.6	1.03 ± 0.19
7	$CaCl_2 \cdot 2H_2O$ [6]		1.05 ± 0.33

[1] Same as in table 2.
[2] Specific activity expressed as mg NH_3-N/100 mg dry kidney/hour.
[3] Basal diet.
[4] Mean ± SE of 4 chicks.
[5] Added as L-glutamic acid hydrochloride.
[6] Provided chloride equal to treatment 6.

TABLE 4

Effects of excess dietary sodium, potassium or the combination on food consumption and growth

Exp. no.	Treatment no.	Dietary additive	Cation[1]/anion	Diet consumed/chick/day	Gain/day
				g	*g*
1,2,3	1	none[2]	1.2	18.0	11.5±0.2[3]
2	10	Na[4](0.297)[5]	1.4	18.1	12.4±0.4
2	11	Na (0.638)	1.6	17.5	11.5±0.3
2	12	Na (0.972)	1.8	17.3	12.0±0.4
2	13	Na (1.311)	2.0	16.4(1)[6]	10.9±0.4[7]
2	14	Na (1.649)	2.2	14.1(3)[6]	9.4±0.3[7]
2	15	Na (1.988)	2.4	14.5(1)[6]	9.1±0.5[7]
2	16	K[4](0.477)[8]	1.4	17.7	11.4±0.3
2	17	K (1.017)	1.6	17.2	11.0±0.5
2	18	K (1.560)	1.8	17.6	10.9±0.3
2	19	K (2.104)	2.0	16.1	9.5±0.4
2	20	K (2.647)	2.2	15.6	9.0±0.3
2	21	K (3.191)	2.4	13.1	7.1±0.3
3	22	Na+K[4,9](0.155, 0.239)[10]	1.4	17.6	11.6±0.4
3	23	Na+K (0.330, 0.508)	1.6	16.9	10.3±0.4
3	24	Na+K (0.506, 0.780)	1.8	18.0	11.7±0.4
3	25	Na+K (0.683, 1.052)	2.0	17.9	11.7±0.3
3	26	Na+K (0.860, 1.323)	2.2	17.1	10.9±0.4
3	27	Na+K (1.036, 1.596)	2.4	17.2	11.3±0.3

[1] Milliequivalents of physiological cation:mEq physiological anion/unit of diet.
[2] Basal diet.
[3] Mean ± SE of 20 chicks/treatment/experiment.
[4] Added as the carbonate.
[5] % sodium added to the basal diet which contained 0.623%.
[6] Number of chicks that died during experimental period; food consumption adjusted.
[7] Mean of live chicks at the termination of the experiment.
[8] % potassium added to the basal diet which contained 0.403%.
[9] Added in equal molar quantities.
[10] % sodium and potassium added, respectively.

the cause for their apparent higher rate of gain. Cation-anion ratios from 1.2 through 1.8 did not depress food consumption or weight gain when either sodium or potassium was added to the diet. A combination of equal equivalents of sodium and potassium (treatments 22–27) added to the basal diet to provide cation-anion ratios equal to either the sodium- or potassium-supplemented diets did not depress food consumption and weight gain at any of the ratios through 2.4. It thus appears that chicks are capable of handling relatively large excesses of dietary cation, providing there is not a preponderance of either sodium or potassium.

The chicks receiving the highest levels of sodium carbonate exhibited symptoms similar to those of chicks receiving a 0.9% sodium chloride solution as drinking water as reported by Selye (11). The symptoms included diarrhea, muscular weakness, gasping respiration, distended abdominal cavity and large pale kidneys. Either edema or "salt effect" was observed in the 5 chicks that died. The latter symptom was characterized by a white pericardium, urates about leg joints and kidneys, and a white granular substance covering the serosal side of the intestine. Scott et al. (4) observed similar symptoms in chicks receiving a diet containing 4% trisodium citrate. Unpublished data from our laboratory indicate that sodium citrate is no more toxic than sodium carbonate.

DISCUSSION

Nesheim and co-workers (2) reported that sodium and potassium were effective in rendering excess chloride innocuous in the chick, whereas calcium and magnesium were ineffective. Calcium, magnesium, sodium and potassium alleviated a growth depression of rabbits receiving a hay diet (12). O'Dell et al. (13) reported

that the same cations restored growth of guinea pigs receiving excess monobasic sodium phosphate. When the dietary anion is phosphate, the role of sodium and potassium is undoubtedly one of neutralization of the excess anion in the kidney tubule. It is more likely that calcium and magnesium act primarily by reducing the amount of phosphate absorbed, as suggested by Forbes and Keith (14).

When the excess dietary anion is chloride or sulfate, sodium, potassium and to some extent magnesium function in the kidney as a neutralizer. Calcium is ineffective in preventing acidosis probably because of the limited capacity of kidneys to excrete calcium or the inability of the intestine to absorb excess calcium.

The response of the chick to a given quantity of acid added to a diet can be predicted based upon the cation-anion ratio of the basal diet. The same appears to be true for the rat. Breuer et al. (15) observed that the supplementation of an amino acid diet containing the Jones-Foster (16) mineral mixture with sodium bicarbonate improved the growth rate of rats. That particular mineral mixture does not contain an appreciable reserve of base. Under conditions of added hydrochloride from arginine, histidine and lysine, the growth-limiting factor was a deficit of absorbable cation. Likewise, the growth of chicks fed a purified diet containing a considerable quantity of hydrochloride from arginine and lysine was improved by the addition of potassium with a metabolizable anion.[3]

High levels of sodium in the diet used in these experiments were less toxic than previously reported (2–4) for diets containing intact proteins. Furthermore, additional dietary potassium has been found to reverse the toxic effect of excess sodium (4). Possible reasons for the differences in susceptibility of chicks to excess sodium are: breed of chick, source or form of dietary nitrogen, or dietary levels of potassium, chloride or sulfate.

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The Role of Iron in the Copper-Zinc Interrelationship in the Rat

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ABSTRACT The effects of dietary supplements of copper (0.02%), iron (0.04%) and zinc (0.75%) on growth and iron metabolism of rats were observed. Although diets with elevated levels of iron significantly lowered uptake, dietary zinc or copper had no significant effect on absorption of orally administered radioactive iron, 96 hours prior to death. Diets high in zinc did, however, significantly lower retention of chronically administered dietary radioactive iron as well as decrease liver iron stores. The data suggest that iron absorbed from the digestive tract was apparently following a normal metabolic pathway after 96 hours, but later deviated significantly under conditions of zinc toxicity. Copper levels were not significantly altered under the conditions of these experiments.

The importance of iron on the interrelationship of copper-zinc metabolism has been recognized. Sivarama Sasty and Sarma (1) state that the apparent antagonism between zinc and copper is a reflection of zinc interference on iron metabolism and that this interference may be a common phenomenon in zinc toxicosis. Magee and Matrone (2) have presented evidence indicating that zinc directly interferes with iron metabolism. They suggest that in addition to copper, iron may be the deficient dietary essential reported by Grant-Frost and Underwood (3) to affect hematopoiesis in zinc-fed rats. The ameliorating effect of copper on zinc toxicity has been viewed by Cox and Harris (4) as being due to the ability of copper to mobilize iron from the liver even when dietary level of iron is at a low level. In any event, there are many aspects of the role of iron in zinc toxicity which are not clear. In view of this, the present study was designed to define more clearly the involvement of iron in the copper-zinc interrelationship and more specifically to observe how these mineral nutrients as dietary additives influence absorption, distribution and excretion of administered radioactive iron.

MATERIALS AND METHODS

The present investigation was divided into 2 experiments. The first was designed to study in rats the effects of various dietary mineral additives upon the distribution and excretion of orally administered ^{59}Fe 96 hours after administration. The second experiment was based on results obtained in the first experiment. It was designed to define more clearly the nature of the iron stores of zinc-toxic rats.

All data were subjected to analysis of variance. Significant differences between any 2 treatment means were determined by using the multiple range test of Duncan as described by Li (5) with some modifications derived from Linquist (6). Data analyzed as percentages were first submitted to an inverse sine transformation (7).

Experiment 1. Seventy-two, 4-week-old, male, rats of the Holtzman strain, weighing an average of 70 g were selected at random and placed in 8 treatment groups of 9 animals each. They were fed their respective rations for 5 weeks and then given 15 uCi of $^{59}Cl_3$ [1] in 2 ml of aqueous solution by stomach tube. After radioisotope administration the rats were placed in Acme Rodent metabolism cages [2] (20 cm × 11 cm × 11 cm). The urine and feces were collected daily until the animals were killed 4 days later. Until ^{59}Fe was given, animals were housed individually in stainless steel wire-mesh cages where food was offered ad libitum from

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[1] Abbott Laboratories, Oak Ridge, Tennessee.
[2] Acme Metal Products, Inc., Chicago.

glass jars with white lacquered screw-cap metal lids having a hole stamped through the middle. Food consumption for each rat was recorded daily; weight gain was recorded 3 times per week throughout the experiment. Distilled, demineralized drinking water was dispensed from glass bottles equipped with stainless steel outlet tubes.

The percentage composition of the basal diet was as follows: starch, 44.4; vitamin-free casein, 18.0; sucrose, 22.4; fiber, 4.0; salt mixture W,[3] 4.0; vitamins [4] in dextrose, 2.2; and corn oil 5.0. Modifications of the basal diet consisted of adding, at the expense of starch, one or more of the following final concentrations of minerals: 0.02% copper as cupric acetate; 0.04% iron as ferric citrate; and 0.75% zinc as the carbonate. These levels of supplementation were used to obtain the following 8 treatments: 1) basal ration (control); 2) Cu; 3) Fe; 4) Zn; 5) Cu + Fe; 6) Cu + Zn; 7) Fe + Zn; and 8) Cu + Fe + Zn.

The rats were killed [5] by exsanguination after being anesthetized with ether to allow blood withdrawal from the inferior vena cava. Desired tissues were removed, weighed and stored at −70° for subsequent analyses.

Radioanalysis using a sodium iodide crystal Auto-Gamma spectrometer system,[6] was conducted on the following samples: 1) liver; 2) kidney; 3) spleen; 4) skeletal muscle; 5) cardiac muscle; 6) duodenum; 7) stomach; 8) bone (right femur); 9) whole blood; 10) plasma; 11) daily urine collections; and 12) daily fecal collections. A counting accuracy of 1% or less was attained during this analysis. Nitrogen determinations by the method of Ferrari (8) were performed on tissues 1 through 6 above. After using the ashing procedure reported by Reitz et al. (9), livers were analyzed for iron by the method of Bandemer and Schaible (10) and for copper as outlined in the AOAC (11).

Experiment 2. Twenty Holzman rats of the same age and sex, and weighing an average of 74 g, were placed in metabolism cages of the type described in experiment 1. One-half of the animals were fed a basal diet; the other one-half were fed a 0.75% zinc diet. Both diets were the same as described in experiment 1 except that $^{59}FeCl_3$ [7] was added to each ration at the rate of approximately 0.02 μCi/g of diet. At the end of 5 weeks each rat was given by stomach tube 40 μCi of carrier-free $^{55}FeCl_3$ [8] in 2 ml of aqueous solution. Four days later one-half the animals in each group were bled and killed as in experiment 1. The other half were killed by exsanguination after which their livers were perfused in situ by inserting a needle into the portal vein and allowing sodium chloride solution (0.9%) to flow until the organ had a pale straw color.

The liver, spleen and right femur were removed from each rat at the time of death. Iron-59 determinations on these tissues and on the urine and fecal collections were accomplished as in experiment 1. In addition, each rat carcass was analyzed for ^{59}Fe utilizing an Armac [9] large-volume scintillation detector. Hemoglobin was determined by the cyanmethemoglobin method outlined by Crosby et al. (12).

A portion of each liver was homogenized in 2 ml of cold distilled water, transferred to a centrifuge tube with an additional 2 ml of distilled water and was brought to a final volume of 8 ml with 4 ml of 20% CCl_3COOH. The resultant mixture was allowed to stand at room temperature for 2 hours and was then centrifuged. Supernatant and precipitated fractions were separately ashed (9), dried and then dissolved in 2 ml of 0.12 N HCL. Portions of these acid solutions were analyzed for total iron (10) and ^{55}Fe. Radioanalysis of ^{55}Fe was accomplished using the scintillation solution described by Bray (13)

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[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75*: 339. (Salt Mixture W, Nutritional Biochemicals Corporaton, Cleveland.)

[4] Vitamin diet fortification mixture in dextrose formulated to give the following ingredients and amounts per kilogram of final mixed ration: vitamin A, 19800 IU; vitamin D, 2200 IU and (in milligrams) α-tocopherol, 110; inositol, 110; ascorbic acid, 990; choline chloride, 1650; menadione, 49.5; *p*-aminobenzoic acid, 110; niacin, 99; riboflavin, 22; pyridoxine 22; thiamine·HCl, 22; Ca pantothenate, 66; folic acid, 1.98; and (in micrograms) biotin, 440; vitamin B_{12}, 29.7. Obtained from Nutritional Biochemicals Corporation.

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

[6] Packard Instrument Company, La Grange, Illinois.

[7] See footnote 1.

[8] Nuclear Science and Engineering Corporation, Pittsburgh.

[9] See footnote 6.

TABLE 1

Weight gain, food consumption, and ^{59}Fe excretion of rats fed rations containing high levels of various mineral additives for a period of 39 days (exp. 1)

	Basal (control)	Fe	Cu	Zn	Fe + Cu	Fe + Zn	Cu + Zn	Fe + Cu + Zn
Weight gain, g/39 days	187	176	177	115**	177	113**	116**	83**
Food consumption, g/39 days	548	545	553	468*	555	441**	452*	402**
Total % of ^{59}Fe excreted in 96 hr:								
Feces	83.6	96.6	81.5	86.1	97.4	84.0	75.5	97.8
Urine	0.062	0.032	0.089	0.047	0.030	0.122	0.094	0.016

* Indicates a difference at the 5% level of significance.
** Indicates a difference at the 1% level of significance.

and a Tri-Carb liquid scintillation spectrometer system.[10]

Another portion of each liver was fixed for 48 hours in neutral buffered 10% formalin after which it was embedded in paraffin by routine procedures. Tissue sections of 5-μ thickness were cut and mounted on clean glass microscope slides. Three different staining procedures were used. Some sections were stained with hematoxylin and eosin, to show the general histology, others by the method of Pearse (14) to demonstrate ferric iron and still other sections by the Turnbull Blue method (14) to determine ferrous iron (which also converts ferric iron to this state). All other conditions and procedures not specifically stated were as described in experiment 1.

RESULTS

Experiment 1. Weight gain; feed consumption. These results are summarized in table 1. Rats of all groups fed rations containing increased levels of zinc, whether in combination with high dietary levels of copper or iron or both, gained significantly less ($P < 0.01$) weight than controls. Animals of these 4 groups also consumed significantly less food — the Cu + Fe + Zn and the Fe + Zn groups at the 1% level of significance; the Cu + Zn and the Zn group at the 5% level.

Radioanalysis; nitrogen analysis: Retained ^{59}Fe, expressed as the percentage of administered ^{59}Fe per gram of tissue (wet weight) 96 hours after administration is also shown in table 2. Since there was a significant difference of body weight at the end of the experiment, it was necessary to correct for this weight difference before statistical analysis. This was done by obtaining for each animal the product of the percentage per gram of concentration and the rat's body weight. A statistical analysis of weighted data revealed levels of significance shown in table 2. Tissues of all rats fed iron-containing rations exhibited lower radioactivity than those of the control group. The presence of dietary copper or zinc apparently had no effect. No other differences were significant. Tissues analyzed for total nitrogen and sta-

[10] See footnote 6.

TABLE 2

Total iron per gram of tissue (wet wt) and percentage of retained ^{59}Fe per gram of tissue (wet wt) 96 hours after administration to rats fed various supplements (exp. 2)

		Basal (control)	Fe	Cu	Zn	Fe + Cu	Fe + Zn	Cu + Zn	Fe + Cu + Zn
1	Liver, %/g	0.354	0.083**	0.454	0.776	0.135**	0.270	0.552	0.144**
		(3.856) [1]	(0.820)**	(4.189)	(4.698)	(1.388)**	(1.748)	(3.428)	(0.735)**
2	Kidney, %/g	0.165	0.040**	0.173	0.306	0.054**	0.093**	0.237	0.071**
3	Spleen, %/g	0.510	0.124**	0.523	0.582	0.143**	0.309	0.781	0.293**
4	Skeletal musole, %/g	0.021	0.005**	0.021	0.026	0.006**	0.013*	0.024	0.006**
5	Cardiac muscle, %/g	0.180	0.031**	0.173	0.324	0.041**	0.096*	0.345	0.084
6	Bone, %/g	0.136	0.038*	0.148	0.230	0.050*	0.076*	0.308	0.100*
7	Stomach, %/g	0.072	0.026*	0.077	0.119	0.035	0.049*	0.108	0.057
8	Duodenum, %/g	0.175	0.060**	0.132	0.185	0.067**	0.080**	0.197	0.081**
9	Red blood cells, %/g	3.668	0.672**	3.414	5.678	0.727**	1.598	6.179	1.849**
10	Plasma, %/g	0.007	0.001**	0.006	0.007	0.002*	0.002**	0.008	0.002**
11	Hematocrit	45.2	44.8	44.2	38.1*	45.1	36.7**	36.4**	38.4**
12	Total of iron, μg/g liver	166	213*	155	82**	170	120*	71**	97**
		(1810)	(2104)*	(1430)	(496)**	(1748)	(777)**	(441)**	(495)**

[1] Values within parentheses represent that amount in entire organ. Value for red blood cells was computed as in table 3.
* Indicates a difference at the 5% level of significance when compared with controls.
** Indicates a difference at the 1% level of significance when compared with controls.

tistically analyzed, after weighting the data in a manner similar to that described above to obtain weighted percentage of administered ^{59}Fe per milligram of nitrogen, yielded essentially the same differences as those shown in table 2. Although fecal excretion of radioactive iron was higher in rats fed iron-containing diets, no significant deviations from normal in urine or fecal excretion of radioiron were observed for any of the experimental groups (table 1).

Iron and copper analyses: Levels of total iron in the liver are shown in table 2. When expressed as micrograms per gram of tissue or total micrograms in the liver, the iron content in rats fed any high zinc-containing ration was lower than normal in all groups. All differences were significant at the 1% level except in the Fe + Zn group. The latter was significant at the 5% level of significance. Iron content in the livers of the Fe group was significantly higher ($P < 0.05$) than normal. No significant between-group differences in liver copper levels were detected.

Experiment 2. Pertinent results of experiment 2 are summarized in table 3. No differences between perfused livers and livers of rats exsanguinated without liver perfusion were noted and are, therefore, treated as belonging to the same respective experimental groups.

Weight gain; feed consumption: As in zinc-fed animals of experiment 1, weight gain and food consumption were significantly lower ($P < 0.01$) than in controls.

Iron-59 analyses: Iron-59 retained in the entire carcass, minus exsanguinated blood, expressed as the percentage of ^{59}Fe consumed was significantly lower ($P < 0.01$) in zinc-fed rats. Tissue concentrations of ^{59}Fe when expressed as the percentage and ^{59}Fe consumed per gram of tissue were significantly lower in the zinc-fed group than in controls. Highest concentrations were found in red blood cells followed by spleen and liver with bone and plasma last. The ^{59}Fe concentration in the precipitated fraction of liver was approximately ninefold higher than in the supernatant in both experimental groups. Expressing the liver ^{59}Fe level as total organ ^{59}Fe content resulted in magnified between-group differences since the

TABLE 3

Weight gain, food consumption and values related directly to organ and tissue content of total iron, "chronically fed" ^{59}Fe and "acutely fed" ^{55}Fe in rats maintained with high zinc diets for a period of 39 days (exp. 2)

	Controls	Zn-fed
1. % of Fe retained in carcass	12.826	6.127**
2. % of ^{59}Fe consumed/g of tissue (wet wt)		
a. liver		
precipitate	0.198 (2.502) [1]	0.064** (0.533)**
supernatant	0.023 (0.291)	0.007** (0.048)**
total	0.221 (2.793)	0.071 (0.581)
b. spleen	0.340 (0.233)	0.151 (0.112)
c. bone (right femur)	0.071	0.043**
d. red blood cells	1.051	0.746*
e. plasma	0.003	0.002*
3. % of ^{59}Fe in carcass/g of tissue (wet wt)		
a. liver	1.758 (22.214)	1.196** (8.981)**
b. spleen	2.652 (1.826)	2.590 (1.920)
c. bone	0.568	0.709
d. red blood cells	9.035	11.348
e. plasma	0.018	0.007**
4. μg of Fe/g of liver (wet wt)		
precipitate	140 (1771)	79** (583)**
supernatant	16 (213)	14 (105)*
total	156 (1984)	93 (688)
5. Disintegrations/min/μg of Fe in liver	358	137**
6. % of ^{55}Fe administered/g of liver (wet wt)		
precipitate	0.421 (5.320)	0.622 (4.671)
supernatant	0.037 (0.468)	0.084 (0.631)
total	0.458 (5.788)	0.706 (5.302)
7. Hemoglobin, g/100 ml blood	15.2	8.2**
8. Weight gain, g	204	125**
9. Food consumption, g	591	488**

[1] Values within parentheses represent that amount in entire organ. Value for red blood cells was computed: $\%/g \text{ in red blood cells} = \frac{\%/g \text{ in whole blood} - [\%/g \text{ in plasma} \times (1 - \text{hematocrit})]}{\text{hematocrit}}$.

* Indicates a difference at the 5% level of significance.
** Indicates a difference at the 1% level of significance.

average liver weight of control animals was 12.636 g, whereas that of zinc-fed rats was 7.509 g.

Iron-59 content when expressed as the percentage of ^{59}Fe within the carcass per gram of tissue and per organ (liver and spleen) are shown in lines 3a – 3e of table 3. A comparison of these values shows that lower levels of radioiron were present in the liver and plasma ($P < 0.01$) of the zinc-fed rats than in the same tissue of the controls.

Total excretion of ^{59}Fe via urine or feces, when presented as the percentage of ^{59}Fe consumed, yielded no significant differences. Fecal excretion of ^{59}Fe was higher in zinc-fed rats; however, within-group variation was such that the between-group difference was not significant.

Iron-55 analyses: Liver concentrations in either the precipitate or the supernatant fractions, expressed as the percentage of administered ^{55}Fe per gram of tissue, were significantly higher ($P < 0.01$) in the zinc-fed animals. However, when corrections were made, as in experiment 1, for the differences in body weight at the end

of the experiment, no significant effects were observed; neither was a significant between-group difference observed in total ^{55}Fe liver content. The ^{55}Fe content in the precipitated liver fraction was higher than in the supernatant by a ratio somewhat similar to that observed for ^{59}Fe.

Total liver iron and hemoglobin levels: The total iron content of livers of zinc-fed rats was approximately one-half that of controls. The concentration of the precipitate fraction was approximately 6 to 9 times higher than in the supernatant fraction in both zinc-fed and control animals. Specific activity calculated as disintegrations per minute of ^{59}Fe per microgram of iron was significantly lower ($P < 0.01$) in the livers of zinc-toxic rats. Hemoglobin levels were significantly lower ($P < 0.01$) in the zinc-fed group.

Histological and histochemical findings: No histological or histochemical differences could be detected utilizing any of the three staining procedures described earlier.

DISCUSSION

The present data indicate that diets high in copper or zinc do not significantly influence gastrointestinal tract absorption, tissue distribution or excretion of a single dose of iron administered orally 96 hours previously. Magee and Matrone (2) have reported the absorption of ^{59}Fe, 48 hours after administration by stomach tube, is not affected by diets high in zinc. In such animals, radioactive iron was increased ($P < 0.05$) in the liver and decreased ($P < 0.05$) in the femurs. In the present study, increased dietary iron resulted in a significantly lower uptake of radioactive iron. The lower uptake was presumably the result of the decreased digestive tract absorption which until relatively recently was thought to be caused by the "mucosal block" mechanism proposed by Hahn et al. (15) and elaborated upon by Granick (16). It is probably due to variations in the activity of a transport system which exists for carrying iron across the mucosal cell membrane (17).

Dietary zinc caused a lower percentage retention of chronically fed radioactive iron, as well as a different relative distribution within the body. It might be assumed that this difference in distribution was caused by lowered liver iron stores in zinc-fed rats. The reduced available iron in the liver, in turn, resulted in lower plasma levels. Sufficiently reduced iron availability would be ultimately evident in lower hemoglobin levels. High dietary zinc apparently did not, however, alter the iron-protein-binding properties of the rats' livers since the protein-bound iron in the liver as indicated by essentially the same ratio of precipitate to total percentage of ^{59}Fe consumed per gram of tissue (table 3, line 2a), was essentially unchanged in the zinc-fed animals.

Why retention of radioactive iron administered by stomach tube, 96 hours prior to death, was apparently unaffected while chronically fed radioactive iron was reduced in zinc-fed rats is difficult to explain. Dissimilarities in the chemical form of the iron within the gastrointestinal tract, could explain a difference and no-difference in uptake of "diet radioactive iron" and "gavaged radioactive iron," respectively, when comparing zinc-fed and control animals. However, if iron absorption has not changed in zinc-toxic animals, it can be concluded that there is a difference in iron excretion. The latter view suggests that iron absorbed from the digestive tract was apparently following a normal metabolic pathway after 96 hours, but later deviated significantly under conditions of zinc toxicity. It is significant to note that the storage rate and site of iron deposition depends not only upon a state of physiological excess, but also upon the chemical form of the iron compound, its origin, and the rate of administration (18–24). For example, iron absorbed from the gastrointestinal tract and soluble iron salts injected in small amounts are stored rapidly and almost entirely in the liver (24); however, following breakdown of red blood cells there is a rapid uptake of iron by the spleen as well as the liver (22, 24) with a later slow distribution of storage iron from spleen to liver (23). Changes in one or more of these metabolic conditions as a result of zinc toxicosis could ultimately result in higher iron excretion.

The lower specific ^{59}Fe activity in the livers of zinc toxic rats chronically fed

^{59}Fe indicates that the total liver iron turnover is slower in the zinc-fed group than in the control group. The distribution of iron between the ferric and hemosiderin liver stores has been reported to remain relatively constant under widely varying total storage levels produced by different conditions (18–20). However, more recently, evidence presented by Cox and Harris (25) supports the contention that liver ferritin is more labile than hemosiderin in zinc toxic rats. From the latter evidence, these authors conclude the major source of iron loss from the liver in zinc toxicosis to be ferritin. Equal iron turnover of liver hemosiderin stores in both normal and zinc toxic animals but a more rapid turnover of ferritin stores in these 2 groups would result in different total liver iron turnover. Control animals with relatively more liver ferritin would have a greater liver iron turnover than zinc fed rats with smaller ferritin stores.

Results of the present investigation indicate that dietary zinc has little effect upon liver copper stores. These observations do not agree with the results of Magee and Matrone (2) but parallel those of other investigators using rats (26) and swine (27, 28). The similarity of tissue copper levels of the present work indicates that a zinc-induced copper deficiency, if it exists in animals under the present experimental conditions, is not due to lowered copper per se but rather to a lack of the physiological availability of the copper present.

Mazur et al. (29) have reported xanthine oxidase participates in the in vivo liberation of liver ferritin iron to the circulation. It could be speculated that the action of zinc was caused by increased activity of xanthine oxidase and consequently an increased rate of iron removal from the liver. This is tenuous, however, since a relationship between iron metabolism and liver xanthine oxidase has not been found (25, 30).

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Potassium and Creatinine as Indexes of Muscle and Nonmuscle Protein in Rats [1]

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ABSTRACT Rate of creatinine formation in vivo was studied in 29 albino rats (12 full-fed males, 10 restricted-fed males and 7 full-fed females) ranging in age from 81 to 188 days. Equations for predicting the quantities of muscle, nonmuscle and total body protein were formulated by using total body potassium and 24-hour urinary creatinine excretion. Predicted values of muscle, nonmuscle and total body protein agree with the gravimetrically determined values within ± 4.17%, ± 7.50% and ± 3.41%, respectively, in rat carcasses.

Total body protein can be separated into muscle protein and remaining body tissue protein. Both of these sources of protein form constant percentages of the fat-free mass (1) and are highly correlated with urinary creatinine excretion (2). In an attempt to predict the amount of these 2 protein components in human and other animals, variations in age, body build and size produce difficulties. In these cases, inconsistencies arise in the ratios of muscle and nonmuscle protein to fat-free mass. Thus, knowing creatinine excretion alone is not adequate in the estimation of body protein.

In view of the above, equations have been formulated in the present study for predicting the amount of muscle and nonmuscle protein and consequently the total body protein in rats. The basis of such predictions rests on studies of potassium: protein and creatine: protein ratios of the muscle and remaining tissues of the body together with the rate of creatinine formation in vivo. When these ratios are accepted, the mathematical treatment is conceptually straightforward. In the present study, predicted values of muscle, nonmuscle and total body protein are compared with the gravimetrically determined values from rat carcasses.

MATERIAL AND METHODS

Studies were made on 22 male and 7 female rats of the Sprague-Dawley strain. All animals at 60 days of age were placed in individual metabolic cages [2] and fed a stock diet [3] ad libitum for 7 days. To obtain a range of body composition, the males were separated into 2 groups. Group A, consisting of 12 rats, was fed ad libitum. Group B, consisting of 10 rats, was full-fed initially and then food consumption was reduced to 50% two weeks prior to body composition analysis. The animals were 81 to 188 days of age when body composition determination was made.

Twenty-four-hour urine specimens were collected for 4 days from all rats prior to body composition determination. Funnels and separators were washed with 0.1 N HCl and rinsed with deionized water daily. The volume of urine collected, together with the wash and rinse water, was measured and constituted the total urine sample. Aliquots of these samples were then taken in duplicate for creatinine analyses by the method of Owen et al. (3).

Procedures for body composition analysis previously described (4) were closely followed. After the carcass was separated into its 5 components, fat (F), water (W), muscle protein (MP), nonmuscle protein (NMP) and mineral (m), potassium and creatine concentrations were determined in each of the tissue components.

Potassium determination: Tissue powder weighing 150 to 200 mg was digested with one milliliter of concentrated HNO_3. After complete digestion, the mixture was

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

[2] Acme Metabolic Cages, Scientific Products, Inc., Denver, Colorado.

[3] Normal Protein Test Diet, Nutritional Biochemicals Corporation, Cleveland.

evaporated to dryness and the residue was dissolved with 10 ml of deionized water. Potassium was determined with a Baird Atomic flame photometer.

Creatine determination: Tissue powder weighing 150 to 200 mg was exhaustively extracted with 10 ml of 0.66 N H_2SO_4. The extract was then autoclaved at 130° for 20 minutes to convert creatine into creatinine which was analyzed by the method of Owen et al. (3). The amount of creatine was obtained by using the factor 1.16 times the amount of creatinine.

Mathematical formulation: The weight of total body protein (P) is assumed to be composed of muscle protein (MP) and nonmuscle protein (NMP), i.e., P = MP + NMP. Studies on tissue analyses (4) verified that total body potassium and creatine are distributed between the muscles and nonmuscular tissues, such that

$$K = K_m + K_n \quad (1)$$
$$C = C_m + C_n \quad (2)$$

where

K = total body potassium (mEq)
K_m = potassium in muscle tissue (mEq)
K_n = potassium in nonmuscular tissue (mEq)
C = total body creatine (mg)
C_m = creatine in muscle tissue (mg)
C_n = creatine in nonmuscular tissue (mg)

The actual determinations of potassium, creatine and protein (4) in these two large groups of tissues revealed the following important proportionality constants.

$K_m/MP = 0.405$ mEq/g
$K_n/NMP = 0.301$ mEq/g
$C_m/MP = 25.49$ mg/g
$C_n/NMP = 6.33$ mg/g

From the ascertained proportionality constants equations 1 and 2 above can be written

$$K = 0.405\ MP + 0.301\ NMP \quad (3)$$
$$C = 25.49\ MP + 6.33\ NMP \quad (4)$$

The quantity of MP and NMP can be stated in terms of K and C by solving equations 3 and 4 simultaneously, whereupon

$$MP = 0.0589\ C - 1.239\ K \quad (5)$$
$$NMP = 4.99\ K - 0.0793\ C \quad (6)$$

Furthermore, NMP can be predicted from the amount of muscle protein and total body potassium by solving equation 3, thus

$$NMP = 3.322\ K - 1.346\ MP \quad (7)$$

From what has preceded, it is clear that the prediction of MP and NMP depends upon K, the amount of total body potassium which is easily assessable, and the unknown quantity of C. If a relationship could be established between the amount of total body creatine and the amount of urinary creatinine excretion ($\dot{Cr}$), so that $\dot{Cr}/C = k$, equation 5 can be rewritten as

$$MP = 0.0589\ \dot{Cr}/k - 1.239K \quad (8)$$

RESULTS

The validity of equation 8 depends on the constancy of k, which is the ratio of $\dot{Cr}/C$, in all 3 groups of rats. The complete individual data obtained in this experiment are presented in table 1. The average k values (0.00787 ± 0.00071, 0.00727 ± 0.00049 and 0.00747 ± 0.00044 for group A, B and females, respectively) for these 3 groups of rats are not significantly different. Therefore, an average $k = 0.00757 \pm 0.00063$ was obtained from all rats.

Substituting 0.00757 for k in equation 8, the resulting equation can be written in the form

$$MP = 7.783\ \dot{Cr} - 1.239\ K \quad (9)$$

The accuracy of equation 9 was tested by comparing the predicted muscle protein weights with those actually determined gravimetrically (fig. 1). The slope of this line, fitted to the predicted and determined values by the methods of least squares, was not significantly different (at the 30% fiducial limit) from unity, and the intercept was not significantly different from zero. This agreement indicates the constants set forth in equation 9 are satisfactory for the prediction of muscle protein over a broad range of body composition. The linear correlation coefficient $r = 0.964$ and a standard deviation of ±4.17% from the mean value were obtained between the predicted and determined values.

The predicted values of nonmuscle protein from either equation 6 or 7 revealed disparities in the relationship with the actually determined values (fig. 2). A rather low correlation coefficient ($r = 0.883$) and relatively large standard deviation (±7.50%) from the mean values were obtained.

Total body protein could be predicted by combining equations 5 and 6 and sub-

TABLE 1

Analytical data

Animal no.	Age	Mass	Fat (F)	Water (W)	Mineral (m)	Total protein (P)	Muscle protein (MP)	Nonmuscle protein (NMP)	Total body potassium (K)	Total body creatine (C)	Urinary creatinine (Cr)	$k \times 10^{-3}$
	days	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*mEq*	*mg*	*mg*	
						Full-fed males						
1	81	307.7	47.84	190.07	10.37	59.41	30.13	29.28	21.37	851	7.090	8.34
2	81	292.3	48.42	175.59	9.60	58.70	27.80	30.90	20.19	851	6.610	7.76
3	109	267.8	28.88	170.81	10.72	57.43	27.57	29.86	19.10	875	6.450	7.36
4	109	351.2	38.16	223.89	13.29	75.89	36.95	38.94	26.41	1121	9.100	8.04
5	130	347.8	53.86	212.01	12.69	69.25	32.50	36.75	23.49	1030	8.020	7.78
6	130	283.7	45.42	170.97	10.42	56.93	26.89	30.04	19.90	863	6.340	7.35
7	145	264.7	46.39	160.41	8.08	49.85	24.17	25.68	17.95	753	5.570	7.40
8	166	360.4	61.04	220.88	11.00	67.43	33.78	33.65	25.06	869	8.330	9.58
9	166	354.2	47.86	229.18	9.88	67.33	33.73	33.59	25.17	957	8.340	8.71
10	166	317.7	35.06	199.80	10.84	72.03	35.09	36.94	23.60	1056	7.800	7.38
11	188	323.7	39.47	208.66	10.38	65.22	31.83	33.39	23.54	1035	7.960	7.69
12	188	374.1	51.35	241.68	10.70	70.32	37.45	32.87	23.07	1186	8.380	7.06
Mean												7.87
SD												0.71
						Restricted-fed males						
1	81	197.9	15.96	134.23	7.47	40.19	19.89	20.30	13.96	644	4.650	7.22
2	88	201.8	13.33	137.60	7.72	43.13	20.28	22.85	15.18	617	5.010	8.12
3	109	231.4	20.12	157.63	8.82	44.83	24.61	20.20	16.86	861	5.790	6.73
4	109	226.8	13.07	154.77	9.33	49.60	23.27	26.33	16.36	840	5.540	6.62
5	130	289.6	32.20	187.34	11.15	55.63	26.04	29.59	19.03	945	7.150	7.57
6	138	262.6	17.83	173.58	11.02	60.13	28.85	31.28	20.80	944	7.060	7.47
7	145	269.9	23.88	180.52	8.14	57.38	26.17	31.21	19.46	925	6.300	6.81
8	145	245.8	24.04	161.14	8.45	52.18	23.63	28.55	18.48	781	5.700	7.29
9	152	287.8	30.51	184.34	11.83	61.08	28.44	32.64	20.87	988	6.930	7.02
10	173	272.4	27.56	179.64	10.38	56.24	27.00	29.24	19.65	901	7.050	7.82
Mean												7.27
SD												0.49
						Females						
1	73	188.4	23.56	119.31	6.88	38.67	18.61	20.06	13.68	587	4.760	8.11
2	87	183.0	24.63	116.79	6.97	34.60	17.24	17.36	12.71	639	4.430	6.94
3	87	197.0	20.23	130.00	7.64	39.17	19.23	19.94	15.07	665	4.770	7.18
4	87	218.4	23.10	142.71	8.83	43.73	21.92	21.81	15.70	762	5.400	7.08
5	101	195.9	25.61	123.46	7.98	38.81	18.55	20.26	14.24	623	4.730	7.59
6	101	242.3	45.81	141.40	8.85	46.20	22.05	24.15	16.20	722	5.730	7.94
7	108	212.1	28.39	133.74	8.12	41.88	21.28	20.26	15.10	698	5.220	7.48
Mean												7.47
SD												0.44

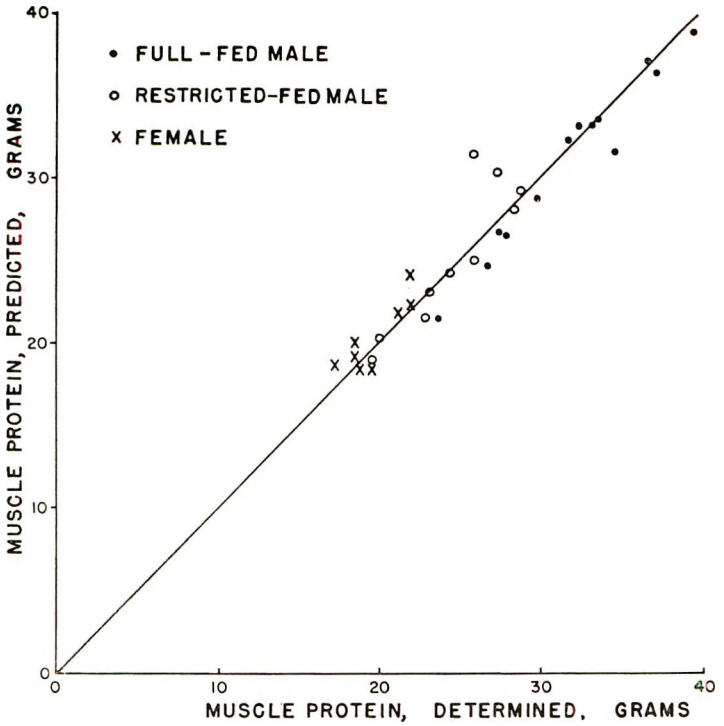

Fig. 1 Agreement between predicted muscle protein and gravimetrically determined muscle protein.

stituting k. The resulting equation could be written as

$$P = 3.751\ K - 2.693\ Cr \qquad (10)$$

The total body protein predicted from equation 10 is similar to that obtained by adding the predicted amount of muscle protein and nonmuscle protein. The predicted quantities of total body protein in all rats were plotted against the gravimetrically determined values as shown in figure 3. A linear correlation coefficient of $r = 0.974$ and a standard deviation of $\pm 3.41\%$ from the mean value were obtained between the predicted and the measured values.

DISCUSSION

To predict the quantities of muscle and nonmuscle protein, and consequently the amount of total body protein, it is necessary that k, the ratio of urinary creatinine excretion to total body creatine, be known and that this ratio be constant. By analyzing the 24-hour urinary creatinine excretion together with the amount of total body creatine in 3 groups of rats (12 full-fed males, 10 restricted-fed males and 7 full-fed females), the k value was derived (table 1). Comparisons of the amount of urinary creatinine excretion and the amount of total body creatine in these groups of rats demonstrated an extraordinarily fixed relationship between these substances over a rather wide range of body composition. No sex differences were apparent.

The average k (0.00757), corresponding to a creatine turnover rate (the amount of creatine excreted as creatinine divided by the total amount of body creatine, $\dot{C}/C$) of 0.88% was unexpected. Thus, reports in the literature (5–7) indicate that the turnover rate of creatine in rats and other species of animals is in the order of 2% per day. However, when the various methods used to determine creatine turnover are considered, this discrepancy is not too unexpected and the lack of validity is striking.

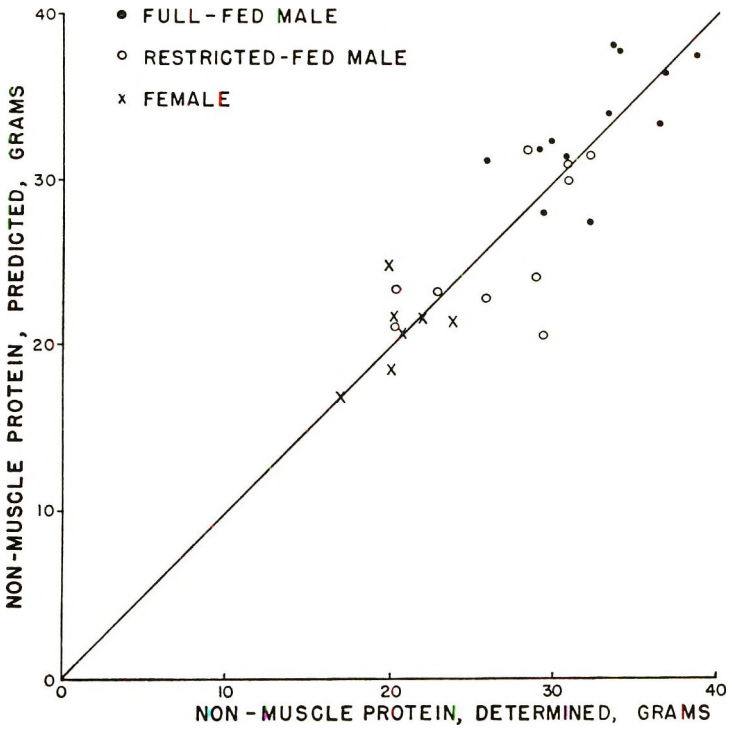

Fig. 2 Correlation between predicted nonmuscle protein and determined nonmuscle protein.

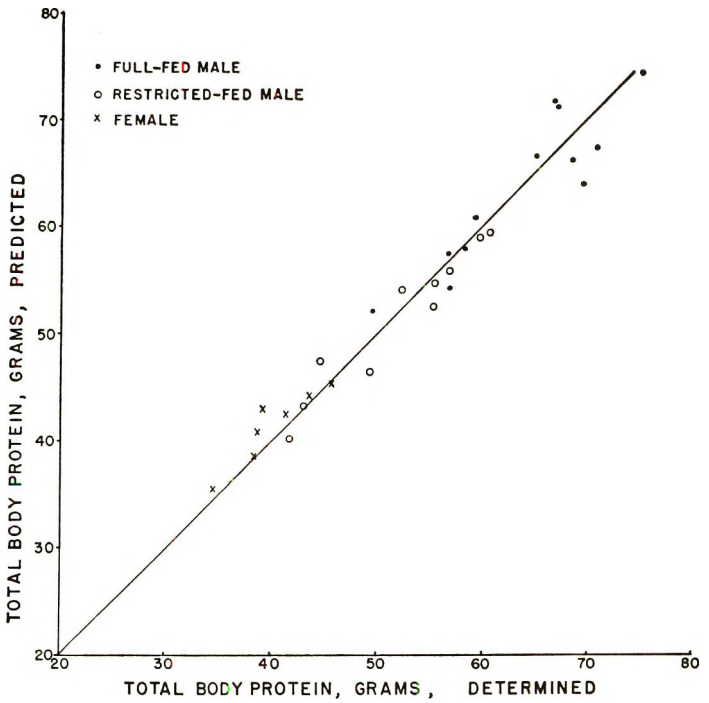

Fig. 3 Comparison of total body protein predicted from the present study with total body protein determined gravimetrically.

Borsook and Dubnoff (6) postulated that the conversions of phosphocreatine and creatine to creatinine follow first order kinetics, and both reactions proceed independently of each other at their individual, but constant rates. At pH 7.0 and normal body temperature, their conversion rates were found to be 0.0011 and 0.00044 mg/hour, respectively. By using the above rates and assuming 60% of the body creatine is normally bound as phosphocreatine, they calculated 2.02% of the total body creatine to be excreted in 24 hours as creatinine. There are, however, reasons to believe that in vivo creatine decreases preferentially to phosphocreatine, since the human or animal body tends to preserve the high energy phosphate stores which are essential to life.

Experiments designed to demonstrate the synthesis and degradation of creatine by use of isotopic creatine (5, 7) appear to confirm that about 2% of total body creatine is converted and excreted as creatinine in urine each day. When the rate of isotopic creatinine disappearance from the experiments of Bloch et al. (5) and of Cohn et al. (7) are studied, large variations are revealed. Bloch et al. (5) had a range from 1.57% to 3.93% (average 2.73±1.02%), Cohn et al. (7) obtained a range from 0.75% to 3.41% (average 1.78±0.95%) for a group of 2 rats and also a range from –0.29% to 3.66% (average 1.45±1.45%) for a group of 3 rats. Although these data are close to the 2% value, statistical methods do not permit a comparison of averages for any interpretation unless the variations are within reliable ranges. Furthermore, the experiments were accomplished by feeding large quantities of isotopic creatine which might have affected the results. In the experiment performed by Bloch et al. (5), rats were fed 53 mg of ^{15}N creatine/kg of body weight for 6 days, and the quantities of creatine ingested by these animals amounted to some 20% of the total initial phosphocreatine-creatine pool. Cohn et al. (7) fed rats 50 mg of isotopic creatine per kg body weight for a period of 21 days, and the total quantities of creatine ingested by these rats amounted to some 50% of the initial pool size.

Borsook and Dubnoff (6), in an analysis of data on 4 species, reportedly calculated approximately 2.5% of the body creatine to be converted and excreted as creatinine each day. When their results were compared with those obtained by more recent workers (8, 9) together with the present report, two important points were revealed. For convenience, the analytical and derived data of all authors are shown in table 2. The overall creatinine coefficient (mg creatinine excreted in urine each day per kg body weight) obtained by previous workers (5, 10, 11) is about 1.6 times higher than values obtained here and by recent workers (8, 9). Such difference may be due partially to the nonspecificity of the Jaffé reaction used in the previous experiments. Since urine has been shown to contain a small amount of noncreatinine chromogens (3), these substances might have increased the optical density of alkaline creatinine picrate procedure. Nevertheless, this method of creatinine determination can account at most for 10% of the increase in creatinine. It may be presumed that the animals used by previous workers were extremely lean and muscular, although it is difficult to prove this convincingly. The possibility that large quantities of exogenous creatine were ingested by these animals appears unlikely since the animals were being fed creatine-free diets. On the other hand, the creatine coefficient (mg of total body creatine per kg body weight) is 1.6 times lower than the value obtained in the present study. This difference appears to result mainly, if not wholly, from the incomplete amount of body creatine reported as total amount of body creatine. Myers and Fine (11) reported the amount of creatine observed in skinned and eviscerated rabbit carcasses as the total amount of body creatine, whereas the values obtained from Chanutin and Kinard (10) were assessed from creatine concentration of muscle with muscle estimated at 40% of body weight. Although results reported by Bloch et al. (5) represent total body analysis, 95% alcohol was used for creatine extraction and this method introduces a possible error due to incomplete extraction of creatine in the animal body since

TABLE 2

Comparison of the mean results of the present experiment with those of other workers

Animal	No. of animals	Weight	Creatinine excretion	Creatinine coefficient	Total body creatine	Creatine coefficient	Reference
		kg	*mg/day*	*mg/kg/day*	*mg*	*mg/kg*	
Rabbit	12	1.749	66.36	37.9	3100	1772	Myers and Fine, 1913 (11)
Dog	11	13.190	464.90	35.3	23000 [1]	1774	Chanutin et al., 1932–3 (10)
Guinea pig	21	0.507	27.42	54.1	1200 [1]	2367	Chanutin et al., 1932–3 (10)
Rabbit	11	2.948	151.49	51.4	6070 [1]	2059	Chanutin et al., 1932–3 (10)
Rat	17	0.298	11.53	38.7	568 [1]	1906	Chanutin et al., 1932–3 (10)
Rat	3	0.611	19.77	32.3	1130	1850	Bloch et al., 1941 (5)
Weighted avg				44.0		2010	
Rat	60	0.227	6.50	28.7			Kumar et al., 1959 (8)
Sheep	65	35.160	1000.00 [2]	28.4			Van Niekerk et al., 1963 (9)
Rat	29	0.268	6.42	24.0	848	3164	Present work
Weighted avg				27.7			

[1] Estimated as follows: average concentration of muscle creatine × 0.40 body weight.
[2] Extrapolated from published graph.

creatine is almost insoluble in alcohol (0.0063 g/100 ml, cold) (12).

In view of the possible errors in the rate constant obtained by previous workers (5, 6, 7, 10), the reported value of 2% appears to be much too high. The present value of 0.88% obtained from three different groups of rats appears more reasonable.

An evaluation of k according to the present experimental procedure has been shown to permit the accurate estimation of muscle, nonmuscle and total body protein in rats from measurements of total body potassium and 24-hour urinary creatinine excretion. The advantage of the present approach is the ability to differentiate the total body protein into its two large components, muscle protein and nonmuscle protein. The assessments of body potassium and urinary creatinine excretion is relatively simple. Furthermore, this approach is independent of the assumed constancy of the fat-free mass, since a small flux of water content from 70% to 73% of the fat-free mass would cause the estimate of body protein to vary some ± 10%.

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Vitamin K Content of Ground Beef [1]

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ABSTRACT The vitamin K content of cooked ground beef was determined by bioassay in the rat to be equivalent to about 0.07 μg of phylloquinone/g of tissue. Approximately one-half of this activity was concentrated from beef fat by molecular distillation. Beef preserved by γ-irradiation did not contain a detectable amount of vitamin K nor any detectable anticoagulant activity.

The first report of readily induced dietary deficiency of vitamin K in a mammalian species appeared in 1959 as the result of a study of the nutritive value of ground beef preserved by irradiation (1). When rats fed irradiated beef were observed to develop hemorrhages, this food became suspect and an intensive study of the etiology of the syndrome was undertaken. Despite the efforts of several laboratories, no toxic anticoagulation due to ingestion of irradiated beef was observed. Johnson and co-workers (2) detected vitamin K in fat extracted from unirradiated beef and estimated that from one-half to three-fourths of the vitamin in ground beef was destroyed during irradiation. Furthermore, Mameesh and Johnson (3) showed that simple nutritional deficiency of vitamin K will result if rats are fed a properly designed diet.

The amount of vitamin K in ground beef is small. Mameesh et al. (4) detected about 35 μg of menadione equivalent per 100 g dry weight. In our laboratory somewhat smaller amounts of vitamin K were observed; however, destruction of this vitamin by γ-irradiation appeared to be the only nutritional factor responsible for the hemorrhagic syndrome. Extensive examination of fresh and irradiated beef by quantitative bioassay in adult male rats revealed no toxic anticoagulants.

METHODS

Coagulation assays. The effect of dietary beef on blood coagulation was quantitatively evaluated by application of a standardized bioassay in adult male rats reported earlier (5). Groups of 10 rats were fed experimental diets for 2 weeks. At the end of each week a sample of blood was taken by cardiac puncture and prothrombin was measured by the method of Hjort et al. (6). Details and statistical aspects of this procedure were discussed earlier (5). Throughout this report coagulation data are expressed as the percentage of normal prothrombin concentration. As determined in earlier studies, single values less than 85% were considered deficient. Means are accompanied by their standard error.

Diets. Beef diets were prepared as described by Metta et al. (1). On a dry-weight basis, they contained 35% ground beef,[2] 35% cornstarch, 19% sucrose, and an adequate supplement of vitamins and minerals except for vitamin K. Vitamin K was added by mixing spectrophotometrically determined amounts of phylloquinone [3] with the lipid components of the diet. Purified diets, prepared as described previously (5), contained 21% protein, 43% cornstarch, 22% glucose monohydrate, 5% corn oil and were also supplemented with vitamins and minerals except for vitamin K.

RESULTS

The assay of diets containing fresh and irradiated beef and the effect of exogenous

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[1] These studies were supported in part under contract and grant with the Office of the Surgeon General, Department of the Army. The opinions expressed are those of the authors and not necessarily those of the Department of the Army.

[2] Beef used in these studies was purchased by the Office of the Quartermaster General. Beef was irradiated at 5.58 megarads by ^{60}Co at the Arco or Savannah River Plants of the Atomic Energy Commission.

[3] Reference to specific forms of vitamin K is made in accordance with the recommendation of the Nomenclature Commission of the IUPAC (7). The K_2-vitamins are menaquinones (abbreviated MK) and vitamin K_1 is phylloquinone (abbreviated K).

TABLE 1

Phylloquinone in diets containing fresh and irradiated beef

Diet [1]	Group no.	Phylloquinone	Prothrombin	Normal values [2]
		μg/g	%	
B-0	1	0	85 ± 3 [3]	9/20
	2	0.005	83 ± 2	10/20
	3	0.010	86 ± 2	12/20
	4	0.025	88 ± 3	15/20
B-6	5	0	60 ± 4	0/20
	6	0.025	77 ± 3	7/20
	7	0.050	96 ± 3	15/20
	8	0.125	102 ± 3	20/20

[1] The diets were as described in the text containing either fresh beef (B-0) or beef irradiated at 5.58 megarads (B-6).
[2] Number of prothrombin concentrations greater than 85%/total observations.
[3] % ± SE.

TABLE 2

Bioassay of alcoholic extracts of beef

Extract [1]	Dietary protein [2]	Prothrombin	Normal values [3]
		%	
B-0	Casein	57 ± 5 [4]	4/20
	Soy	43 ± 3	0/20
B-6	Casein	63 ± 5	4/20
	Soy	37 ± 4	0/19

[1] Total lipid-free alcoholic extract of 2.27 kg of fresh (B-0) or irradiated (B-6) beef incorporated into 3 kg of diet.
[2] 21% protein in the purified diet described earlier (5).
[3] Number of prothrombin concentrations greater than 85%/total observations.
[4] % ± SE.

vitamin K are shown in table 1. The best estimate of the difference in vitamin K activity between the 2 diets is 0.025 μg/g. This is based on a favorable comparison between groups 1 and 6 and again between groups 4 and 7. During subsequent studies the difference between fresh and irradiated beef was accounted for by bioassay of separated fractions of the meat.

In earlier experiments, the protein portion of the diet was separated by exhaustive extraction of cooked ground beef. Rats fed a vitamin K-deficient diet containing protein from fresh beef were more deficient than those fed protein from irradiated beef (5). The difference was small but represented a vitamin K equivalent of about 0.05 μg/g in favor of irradiated beef. In the present study, the alcoholic fraction remaining after purification of the protein and removal of the lipid fraction was also assayed. The alcohol was evaporated in a non-foaming rotary spray evaporator [4] and the residue assayed after mixing in purified diets containing either casein or soy protein (table 2). Comparison of those groups of rats fed extract from fresh or irradiated beef revealed no significant difference in prothrombin levels; thus this fraction of ground beef contained no component which contributed to the difference in vitamin K nutrition between fresh and irradiated beef.

Essentially all of the estimated difference in vitamin K between fresh and irradiated beef was observed in lipid fractions prepared in the following manner. Meat was autoclaved on stainless steel grills and the resulting drippings were centrifuged to yield about 60% of the fat present in whole beef. The remaining aqueous fraction and the cooked solids were dehydrated with alcohol and thoroughly extracted with petroluem ether to remove the residual fat. The aqueous alcoholic fractions were partitioned against the petroleum ether fractions and the petroleum ether was finally removed under reduced pressure. All steps were carried out in subdued light. The total fat obtained by this procedure was assayed in the standard purified diet (5). As shown in table 3, vitamin K was detected in fresh beef fat but not in fat from irradiated beef. Based on dose-response data reported earlier (5), the diets containing fat from fresh beef contained about 0.02 μg of vitamin K/g. This is 0.4 μg/g of fat or 0.07 μg/g of fresh tissue (18% extractable lipid).

In early experiments with beef fat we were unable to detect vitamin K by chick

[4] Nester and Faust Manufacturing Corporation, Newark, Delaware.

TABLE 3

Bioassay of beef fat in adult male rats

Lipid [1]	Dietary protein	Prothrombin	Normal values [2]
		%	
B-0 fat	Casein	96±3 [3]	15/19
B-6 fat		79±4	9/20
Corn oil		81±3	19/40
B-0 fat	Soy	52±3	0/20
B-6 fat		47±3	0/20
Corn oil		46±3	1/40

[1] Standard purified diet containing 5% of the indicated lipid from fresh (B-0) or irradiated (B-6) beef. Data with corn oil are included for comparison from a previous report (5).
[2] Number of prothrombin concentrations greater than 85%/total observations.
[3] % ± SE.

TABLE 4

Molecular distillation of vitamin K

Oil [1]	Weight	Distillate	Vitamin K [2]
	g	*g*	*μg*
Corn oil	920	80	none
Corn oil + 800 μg vitamin K	900	80	800
B-0 fat	1000	120	200
B-6 fat	910	140	none

[1] Fresh (B-0) and irradiated (B-6) beef fat prepared as described in the text. The distillates of these fats made up approximately 3% of previously described diets (8).
[2] Determined by chick bioassay (8) and expressed as phylloquinone.

bioassay; however, the activity was detected after concentrating the vitamin by molecular distillation [5] (table 4). The distillate from 1000 g of fat from fresh beef contained 200 μg of vitamin K but a similar preparation from irradiated beef gave no evidence of vitamin K. The first 2 experiments shown in table 4 show the recovery of phylloquinine added to corn oil. A distillate representing about one-tenth of the original oil contained all of the added vitamin. Based on the data in table 4, fresh beef contains about 0.04 μg of distillable vitamin K/g.

DISCUSSION

The occurrence of the biological activity of vitamin K in the fat-soluble portion of ground beef provides biological support for the identification by Martius (9) of menaquinone-4 among the metabolites of vitamin K in non-hepatic tissue. More generally, it supports the concept that vitamin K does occur in non-hepatic tissues where it cannot directly affect prothrombin synthesis.

The difference in vitamin K activity between fresh and irradiated beef resulted essentially from the occurrence of vitamin K in fresh beef. This substantiates the view that anticoagulants due to irradiation do not play a role in this syndrome. The quantitative aspects of these studies demonstrate that extremely small amounts of vitamin K may be required to normalize prothrombin concentrations in the rat. As little as 0.05 μg of phylloquinone/g of diet protected rats fed irradiated beef, whereas the unsupplemented diet produced a marked deficiency. By comparison, rats fed fresh beef were less deficient but also required some supplemental vitamin K to maintain normal plasma prothrombin. These data emphasize that a nutritional deficiency of vitamin K may occur in mammalian species without the appearance of clinical symptoms of hemorrhage or even greatly elevated prothrombin times. The important role of nutrition in the regulation of anticoagulation in man has recently been studied (10).

Vitamin K was not detected in irradiated beef. The concentration of vitamin K in fresh ground beef was approximately 0.07 μg/g. Slightly more than half of this activity was recovered by molecular distillation. This is lower than expected for phylloquinone or menaquinone-4 but predictable for naphthoquinones of higher molecular weight.[6] Larger homologs of vitamin K have been recovered recently from beef liver (11) and may also occur in non-hepatic tissue.

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[5] CMS-5 centrifugal molecular still, Consolidated Vacuum Corporation, Rochester, New York.
[6] In separate experiments, quinones as large as ubiquinone-10 added to corn oil were not recovered by molecular distillation.

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Effects of Zinc Deficiency per se and of Dietary Zinc Level on Urinary and Endogenous Fecal Excretion of ^{65}Zn from a Single Intravenous Dose by Ruminants [1,2]

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ABSTRACT Following single intravenous doses of ^{65}Zn, rate of excretion was studied in experiments designed to separate the physiological effects of a zinc deficiency per se from those of level of dietary zinc. Fecal excretion of ^{65}Zn was higher in normal animals fed a normal diet containing 46 ppm zinc then in comparable animals fed a zinc-deficient diet containing 6 ppm zinc. Specific activity of fecal zinc from animals fed the deficient diet was much higher, reflecting the much lower level of total fecal zinc. When fed the same diets, normal animals excreted more ^{65}Zn in the feces than did zinc-deficient animals. However, the deficient animals excreted more ^{65}Zn in the urine, suggesting a possible pathological effect of the deficiency on the kidneys. Level of dietary zinc per se did not affect urinary excretion in normal animals. Urinary excretion of ^{65}Zn was highest the first day, decreased sharply the second day, and decreased gradually thereafter. Excretion of ^{65}Zn via urine was low, with the total for 13 days being less than 0.3% of the dose. Fecal excretion was highest the second and third days after dosing and decreased gradually thereafter. Level of ^{65}Zn in the blood declined very rapidly immediately following dosing and very slowly after the first day. These studies indicate that both a low zinc diet and a zinc deficiency per se cause reduced endogenous fecal excretion of ^{65}Zn for at least 2 weeks after the zinc enters the blood, thus contributing to homeostasis of this element.

The effects of a zinc deficiency on ruminants have been described in several publications (1–7). However, very few studies are available concerning metabolism of ^{65}Zn by ruminants (8, 9). In order to determine the true absorption of zinc the level of endogenous excretion must be known. For an understanding of endogenous excretion of zinc, it is important to know when the excretion occurs after the zinc enters the blood.

These experiments were conducted to determine the effects of a zinc deficiency and level of dietary zinc, on endogenous fecal and urinary excretion of ^{65}Zn by ruminants with time after a single intravenous dosing. It was considered important to separate the physiological effects of the zinc deficiency per se from the influence of a dietary deficiency of zinc at the time of dosing.

EXPERIMENTAL

Two experiments were conducted using tracer doses of ^{65}Zn to study endogenous excretion of ^{65}Zn with time after a single intravenous dose. In the first, the effects of a zinc-deficiency per se on endogenous fecal and urinary ^{65}Zn excretion were determined using the same zinc-deficient diet for both deficient and normal animals at the time of the metabolism study. In the second experiment, effects of a normal and a zinc-deficient diet on ^{65}Zn metabolism were studied in normal animals.

Experiment 1. Two male goats were fed a zinc-deficient diet for several weeks, and two comparable animals were fed the same diet except with zinc added. Prior

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[2] Appreciation is extended to the Kraft Foods Company, Garland, Texas, for the dried whole whey; to the Chas. Pfizer Company, Terre Haute, Indiana, for antibiotics and vitamins; to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the biotin; to the Commercial Solvents Company, New York, for the choline; to Basic Incorporated, Cleveland, for the magnesium oxide; and to the Allied Chemical Company, Atlanta, Georgia, for the urea.

to the experiment the animals were fed practical-type diets including milk replacer and calf starter until they were 4 months old.

The zinc-deficient basal diet consisted of the following per 100 kg: glucose monohydrate, 19.5 kg; cornstarch, 25.0 kg; dried whole whey (spray process), 20.0 kg; cellulose, 10.0 kg; gelatin (flake, 50 bloom), 10.0 kg; egg albumin (autoclaved), 3.0 kg; urea (feed grade, 42% N), 0.5 kg; $KHCO_3$, 1.5 kg; $NaHCO_3$, 2.5 kg; dicalcium phosphate (anhydrous, food grade), 2.0 kg; $CaCO_3$ (marble dust), 1.0 kg; lard (stabilized), 3.0 kg; Na_2SO_4 (anhydrous), 350 g; KCl, 550 g; NaCl, 484 g; MgO (56% Mg),[3] 165 g; $Fe_2(SO_4)_3 \cdot XH_2O$, (20% Fe by assay), 22 g; $MnSO_4 \cdot H_2O$, 4.4 g; $CuSO_4$, 3.1 g; $CoCO_3$ (45–50% Co by assay), 22 mg; KI, 18 mg; thiamine·HCl, 0.9 g; riboflavin (USP), 2.0 g; Ca pantothenate, 3.3 g; pyridoxine·HCl, 1.1 g; nicotinic acid (USP), 2.2 g; folic acid (USP), 0.22 g; cyanocobalamin (1 mg vitamin B_{12} activity/g), 2.2 g; menadione sodium bisulfite (63%), 0.33 g; D-biotin, 26 mg; *d*-α-tocopheryl acetate (333 IU vitamin E activity/g), 2.2 g; vitamin A palmitate (250,000 IU/g), 17.6 g; vitamin D_3 (200,000 IU/g), 2.2 g; choline·Cl (70%), 264 g; and oxytetracycline (25%), 88 g. By analysis (10) the basal diet contained 6 ppm zinc on a dry-matter basis. The control purified diet was identical except for the addition of 40 ppm of supplemental zinc as ZnO.

After those animals that were fed the zinc-deficient diet developed typical clinical symptoms of a zinc deficiency (1, 2, 4, 5), they and the two comparable controls were accustomed to metabolism crates for 1-week prior to a single intravenous dosing and sample collections. To avoid confounding the effects due to differences in zinc level with those attributable to a zinc-deficiency in the animal, the normal animals were fed the zinc-deficient diet beginning one week before dosing. Each goat was restricted to and consumed 500 g of the diet per day while in the metabolism crates. The animals were fed once per day and given water ad libitum. Frequent clinical examinations were made by personnel of the School of Veterinary Medicine to be certain that the zinc-deficient animals maintained typical symptoms of the deficiency and that the controls were normal throughout the study. Clinical symptoms are the most reliable and sensitive method, currently available, of positively determining that a specific experimental calf or goat is zinc-deficient.[4]

When dosed with 550 μCi of ^{65}Zn each of the goats weighed 21 kg and were 5.5 months old. The feed intake of the controls was restricted to the extent that all animals were the same weight. The ^{65}Zn (specific activity, about 5,000 mCi/g of zinc) was in the form of $ZnCl_2$ in HCl solution. The ^{65}Zn solution was adjusted to pH 5 with NaOH crystals and injected into the jugular vein with a syringe through a plastic catheter which had been placed in the vein immediately before the injection.

Total fecal and urine collections were made daily and blood samples were taken from the jugular vein at periodic intervals. ^{65}Zn content of blood and urine samples was determined in test tubes with a deep-well scintillation counted with a sodium iodide crystal. The ^{65}Zn activity of the feces was determined with a whole-body counter and a 400 channel multichannel analyzer[5] designed for small animals or organs and tissues. Portions of the dosing solution of ^{65}Zn were used as standards in counting. The standards were comparable in size and geometry to the samples. Total zinc concentration was determined by atomic absorption spectroscopy (10) with nitric-perchloric-sulfuric acid wet-ashing of samples.

Experiment 2. Two Holstein and 4 Jersey male calves were fed a practical-type diet including milk replacer and calf starter until 3 months of age. They were then adjusted to the control purified diet described above. One and one-half weeks prior to a single intravenous dose of 550 μCi of ^{65}Zn per calf, three of the calves (one Holstein and 2 Jerseys) were given the zinc-deficient diet described above and the other three continued to be fed the control diet. On an average, the Holsteins and Jerseys weighed 117 kg and 67 kg, respectively. The Holsteins were fed 2270

[3] Sold under the name of Magox, feed grade (guaranteed to contain 56% Mg).

[4] Unpublished data, W. J. Miller, D. M. Blackmon and J. D. Morton, 1966.

[5] The whole-body counter was manufactured by Metrix, Inc., Deerfield, Illinois, and the multichannel analyzer by Technical Measurements, Inc.

and the Jerseys 1362 g/day in 2 equal daily feedings. At the time of killing, 2 weeks after dosing, all of the animals were clinically normal. The data on ^{65}Zn content of the blood were adjusted to compensate for the deviation in individual body weights from the average. This put the data on a basis that would be equivalent to each calf having been given 6.5 μCi of ^{65}Zn/kg of body weight. All other aspects of the procedure in this experiment were the same as in experiment 1.

RESULTS AND DISCUSSION

Normal animals excreted an appreciably higher percentage of the intravenous dose of ^{65}Zn in the feces than did those suffering from a zinc deficiency (figs. 1-A and 1-B) (exp. 1). To avoid confounding the dietary effects of level of zinc in the diet with the effects of the zinc deficiency per se, the normal animals were fed the deficient diet beginning one week prior to dosing. Although no clinical symptoms of a deficiency were observed in these control animals at any time, it is possible that the deficient diet had resulted in some borderline effects before the animals were killed 5 weeks after feeding of the deficient diet began. This may have been partially responsible for the fecal excretion of ^{65}Zn not being measurably different between the normal and the deficient animals after 17 days following dosing. Results from each of the animals on the same treatment were in good agreement. For example, total excretion of ^{65}Zn for the first 17 days following dosing was 6.6% and 6.8% of the dose for deficient animals and 8.3% and 9.6% for the controls.

Endogenous fecal excretion of ^{65}Zn following an intravenously administered tracer dose was consistently lower in normal animals fed a zinc-deficient diet containing 6 ppm zinc than in comparable animals fed the normal diet containing 46 ppm zinc (figs. 2-A and 2-B) (exp. 2). Thus, both the zinc deficiency per se and the zinc-deficient diet reduced endogenous fecal excretion of ^{65}Zn for at least 2 weeks after the ^{65}Zn was injected into the blood stream. In these experiments the magni-

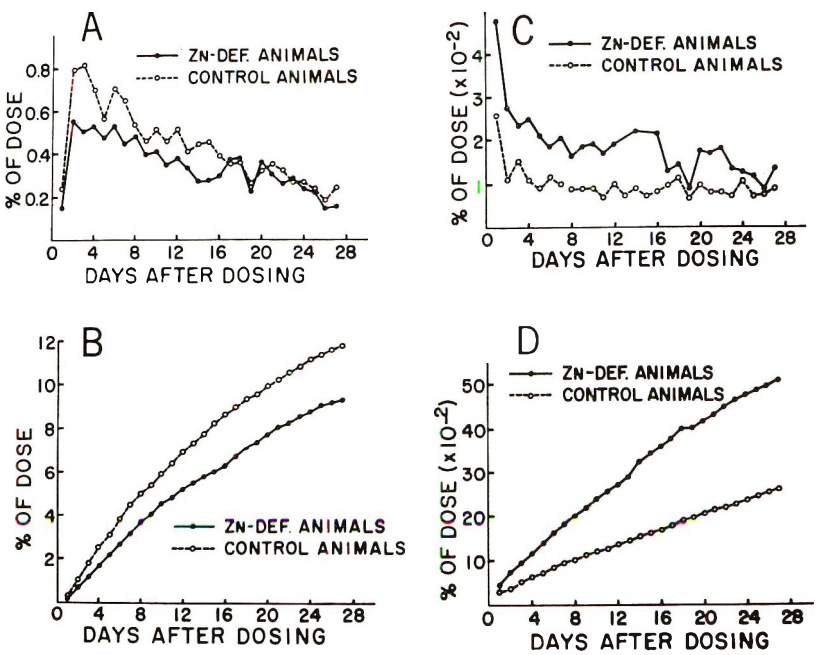

Fig. 1 Effects of zinc deficiency per se in goats fed a zinc-deficient purified diet (6 ppm zinc) on ^{65}Zn excretion following a single iv dose: (A) daily rate of fecal excretion; (B) accumulated total fecal excretion; (C) daily rate of urinary excretion; (D) accumulated total urinary excretion (exp. 1, 4 animals).

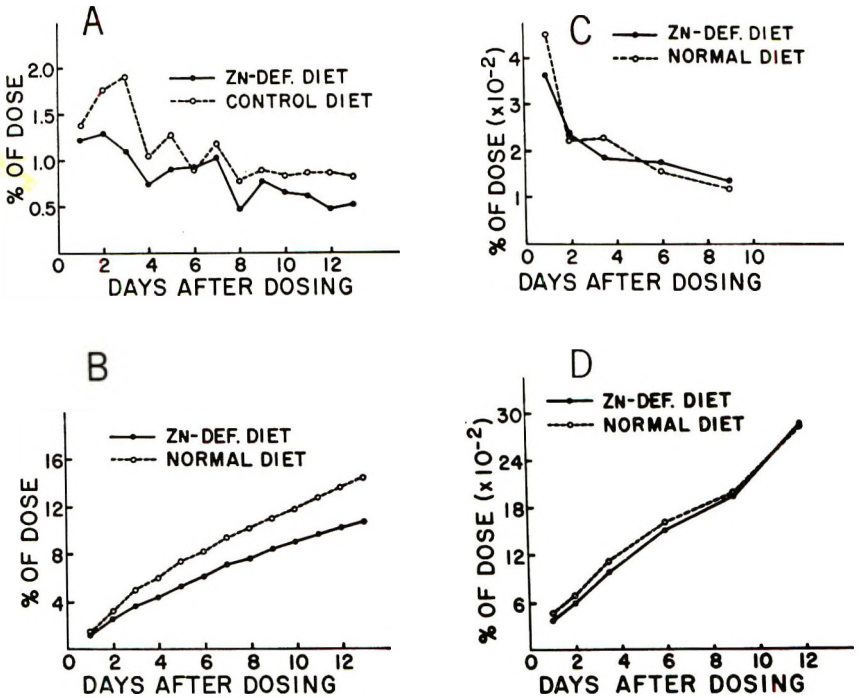

Fig. 2 Effects of level of dietary zinc (6 vs. 46 ppm zinc) for normal calves fed a purified diet on ^{65}Zn excretion following a single iv dose: (A) daily rate of fecal excretion; (B) accumulated total fecal excretion; (C) daily rate of urinary excretion; (D) accumulated total urinary excretion (exp. 2, 6 animals).

tude of the 2 effects was approximately comparable. Whether the 2 effects would be additive has not been determined, as experiments in which the dietary and physiological effects were confounded and compared with unconfounded treatments have not been conducted.

The specific activity (% of the ^{65}Zn dose excreted/g of zinc) of the zinc excreted via feces was somewhat higher for the zinc-deficient than for the normal animals (fig. 3-A) (exp. 1). The higher level of ^{65}Zn (as a percentage of the dose) from normal animals represents increased endogenous excretion. The lower specific activity of fecal zinc from normal animals is owing to the level of stable fecal zinc being twice as high as in feces from deficient animals. The lower level of stable zinc in feces of deficient animals is both a reflection of reduced endogenous zinc and of higher true absorption.

The specific activity of the fecal zinc from normal animals fed a zinc-deficient diet was far higher than that of comparable animals fed the diet with a normal level of zinc (fig. 4-A). This is predominately a reflection of the much higher level of fecal zinc in calves fed the normal diet.

The highest rate of fecal excretion of ^{65}Zn occurred during the first few days following dosing and declined slowly for the remainder of the collection periods (figs. 1-A and 2-A).

Data from a preliminary experiment indicated that the level of ^{65}Zn in the blood dropped very rapidly immediately following dosing (fig. 5). Although samples were not obtained in experiment 1 until 24 hours after dosing, 98.9 and 98.5% of the dose had disappeared from the blood of zinc-deficient and control animals by this time. For these calculations, blood volume was assumed to be 6% of body weight (11). Between the first and the twenty-seventh days following dosing, the level of ^{65}Zn in blood declined 28% in zinc-deficient animals and 39% for normal animals fed the same diet (fig. 6-A). This

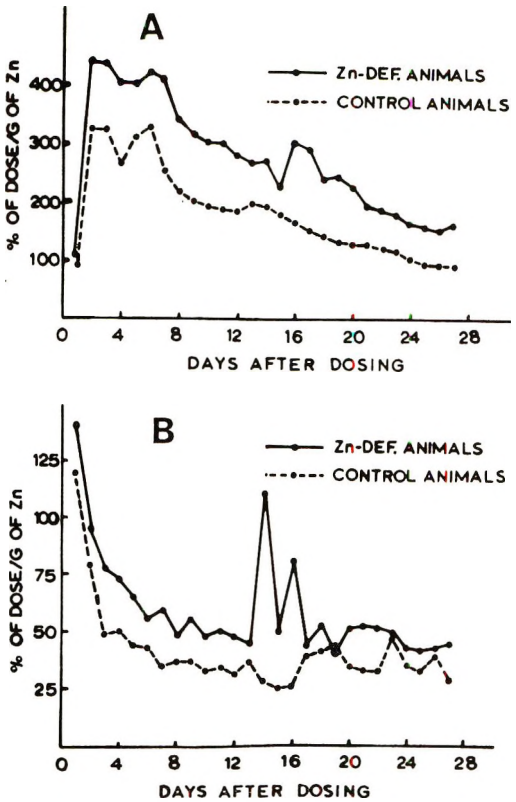

Fig. 3 Effects of zinc deficiency in goats fed a zinc-deficient purified diet on specific activity of zinc (% of ^{65}Zn dose excreted/g of total zinc excreted) following a single iv dose: (A) specific activity in fecal zinc; (B) specific activity in urinary zinc (exp. 1, 4 animals).

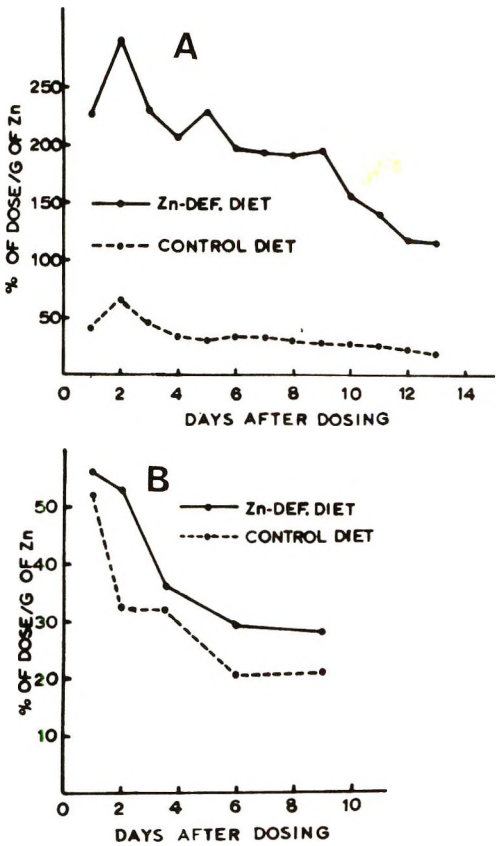

Fig. 4 Effects of level of dietary zinc (6 vs. 46 ppm zinc) for normal calves fed a purified diet on specific activity of zinc (% of ^{65}Zn dose excreted/g of zinc excreted) following a single iv dose: (A) specific activity in feces; (B) specific activity in urine (exp. 2, 6 animals).

difference was not significant statistically. In zinc-deficient animals the decline was greater the first day after dosing than in normal animals (fig. 6-A). However, subsequent to this, the rate of decline was less rapid in zinc-deficient animals, and hence the level of ^{65}Zn was approximately the same in both groups 27 days following dosing. Level of dietary zinc fed to normal animals did not influence ^{65}Zn content of the blood (fig. 6-B).

The highest level of urinary excretion of ^{65}Zn occurred the first day after dosing (figs. 1-C and 2-C). On the second day the amount was approximately one-half that of the first day. Following this the rate of decline was much slower. These changes appear to reflect ^{65}Zn content in the blood to some extent. However, it is known that the distribution of ^{65}Zn between plasma and red blood cells is changing during these times (12, 13).[6]

Level of dietary zinc did not affect urinary excretion of ^{65}Zn by normal animals (figs. 2-C and 2-D). This is in agreement with the conclusion of Vallee (14) indicating that diet does not affect urinary excretion of zinc. On an average, the specific activity of the zinc was somewhat lower in the urine of the control calves (fig. 4-B). However, this was largely the result of the higher level of zinc in the urine of one control calf. Urinary zinc excretion increased when rats were fed diets containing very high levels of zinc (5,000 ppm) (15). However, this is a very differ-

[6] Unpublished data, W. J. Miller, R. P. Gentry, D. M. Blackmon, W. J. Pitts and G. W. Powell, 1966.

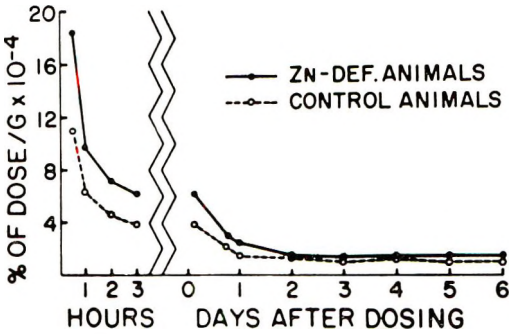

Fig. 5 ^{65}Zn content of whole blood following single iv doses in one zinc deficient and one control animal fed a zinc-deficient purified diet. Data from preliminary experiment presented to show rate of ^{65}Zn decline immediately after dosing.

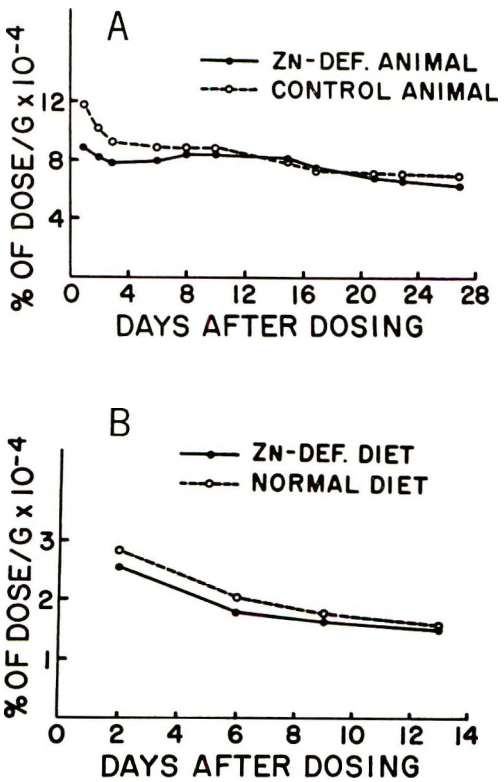

Fig. 6 ^{65}Zn content of whole blood following single iv doses: (A) effects of zinc deficiency per se in goats fed a zinc-deficient purified diet in experiment 1, 2 animals per treatment; (B) influence of normal (46 ppm) and zinc-deficient (6 ppm) levels of zinc in normal calves of experiment 2 (6 animals).

ent situation from the present study in which the control diet contained 46 ppm or less than 1% as much as was fed the rats. It is possible that effects of the very high levels of zinc may have resulted from some mechanism relating to its toxic effects. Cattle fed intermediate levels of zinc (1,000 ppm) did not excrete more zinc via urine than those fed a normal diet (8).

Urinary excretion of ^{65}Zn was twice as high in zinc-deficient as in normal animals (figs. 1-C and 1-D). Specific activity of the zinc was somewhat higher in the urine of the zinc-deficient animals but not as much higher as the increase in level of ^{65}Zn excreted reflecting a somewhat lower level of zinc in the urine from the zinc-deficient animals (fig. 3-B). However, this is in contrast with results from other experiments with zinc-deficient animals and suggests that perhaps the zinc-deficiency does not have very much effect on the total zinc excretion via urine.

Total urinary excretion of ^{65}Zn was quite low. From the standpoint of zinc balance to the animals the urinary loss is normally of minor importance. This is in agreement with previous work in several species of animals (8, 9, 12, 13, 15). The accelerated urinary excretion of ^{65}Zn by zinc-deficient animals suggests the possibility of a pathological alteration. It is opposite to the effect on fecal excretion and is in conflict with homeostatic conservation of the mineral which appears to be in effect, overall, with zinc-deficient animals.[7]

In the 2 experiments, different species of ruminants were utilized. The design of the study is such that it is not possible to determine whether the 2 species would metabolize ^{65}Zn identically. Nevertheless, in general, the same principles should apply to both. The zinc deficiency syndrome is essentially the same in both species (1–6). However, the time required for maturity is quite different. Since age appears to have an effect on several aspects of zinc metabolism, it is probable that some quantitative differences would appear due to stage of maturity and other factors which would usually be different should the 2 species be compared.

[7] See footnote 6.

The present experiments show that either a zinc deficiency or a reduced level of dietary zinc results in a consistent reduction in level of endogenous ^{65}Zn for a considerable time after the isotope enters the blood stream. However, the data do not provide information to indicate the average level of endogenous ^{65}Zn that would be excreted under steady state or equilibrium conditions. Before the understanding of endogenous zinc excretion is regarded as being relatively complete, it will be desirable to study the relationship of the specific activity of ^{65}Zn in the plasma and feces to determine whether the same relationships hold as for ^{45}Ca and ^{32}P in the steady-state condition (16, 17). If the assumptions required in the classic methods of determining average endogenous excretion of certain other minerals under steady state conditions (16, 17) can be established as valid for zinc, such studies could contribute appreciably to the understanding of zinc metabolism in ruminants.

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Guide for Authors

(*Revised November 1962*)

New policy concerning page charge, abstracts and summaries, and numerical citation of literature

The Journal of Nutrition welcomes concise reports of original research bearing on the nutrition of any organism. Preliminary notes, or papers already published or in press elsewhere are not acceptable. Consideration will be given to the publication of supplements at the author's expense.

Manuscripts are to be typewritten on bond paper (8½ × 11 inches). Double spacing should be used throughout, including that on title page, tables, legends, footnotes and references. A margin of about 1¼ inches (or 3 cm) is needed at each side of the sheets.

An abstract of 200 words or less, typed on a separate sheet and double-spaced, should be included. When published, this will appear ahead of the introductory section of the text in lieu of a summary at the end. The abstract should present the scope of the work and the principal observations.

An original and a carbon copy of the manuscript and abstract should be sent *flat* by registered mail to

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THE JOURNAL OF NUTRITION
GRADUATE SCHOOL OF NUTRITION
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The following information should be listed on page 1:

1. Complete title of paper (in upper and lower case letters).
2. Name or names of authors.
3. Institution or laboratory, with city and state.
4. Shortened form of title (not more than 48 letters and spaces).
5. Address and name of author to whom proof is to be sent.

(*All footnotes*, including those pertaining to the title page, should be placed on a separate sheet, typed double-spaced.)

The text should begin on page 2. The manuscript should be prepared in complete and finished form. Number all pages consecutively in the following order: title page, text, literature cited, footnotes, abstract, tables and figure legends. *Never divide or hyphenate a word at the end of a line*, and do not staple together the pages of the manuscript.

Start each of the following sections on a new page, typed double-spaced:

1. The abstract.
2. Tables, with appropriate heading (a separate sheet for each table).
3. Explanation of figures, numbered and listed consecutively (do not affix illustrations to these sheets).
4. Footnotes — including those referring to title, author, institution and text.
5. Literature cited, arranged numerically in the order of first citation in the text, as "Jones and Smith (1) and others (2) have reported . . . ."

LITERATURE CITED

1. Jones, K. Z., and X. Y. Smith 1972 Growth of rats when fed raw soybean rations. J. Nutr., *95*: 102.
2. Brown, Q. R., V. A. Ham and I. V. Long 1971 Effects of dietary fat on cholesterol metabolism. J. Nutr., *94*: 625.

The metric system is used for all units, and temperature is expressed in the centigrade scale. The words "et al.," "per se" and "ad libitum" are not underscored. The repeated use of a unit in a sentence may be avoided by stating the common unit used, as "the diet consisted of the following: (in grams) sucrose, 50; casein, 130; . . ." Or, in other instances, as "8, 12 and 14%." The expression "*fed* a diet" is preferred to "*on* a diet." Certain other preferred usage is described in the *Style Manual for Biological Journals*.[1] The use of laboratory jargon is to be avoided, as well as such comments as "it can be seen that," " it is interesting that," and "it can be noted that." The word "quite" is often misused. Use of the active voice in all writing is preferred to the passive voice.

Registered trade names. Registered trade names are not used in text, tables or figures, except when necessary to identify certain equipment. A trivial or descriptive name should be used in text and tables, with a footnote giving the registered trade name, manufacturer and address (city and state).

Footnotes. In the text, footnotes should be numbered consecutively, including any indicated on the title page. For tables, footnotes should be typed *double-spaced* directly beneath the table, and numbered 1, 2, 3, etc. Superscripts in the table should appear consecutively, starting at the top of the table and reading from left to right across each line. Footnotes to tables are independent of the other footnote numbers in the text. Symbols are not used as superscripts and subscripts.

Acknowledgments. Financial support should be listed as a footnote to the title. Credit for materials should be listed as a footnote in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will appear at the end of the text.

Literature Cited. Accuracy and adequacy of the references are the responsibility of the author. Literature cited should be checked carefully with the original publication. References to abstracts of verbal reports and to other unedited material (as the abstracts in Federation Proceedings, theses, and industrial technical bulletins) should be treated as text footnotes. Reference to a paper in press may be included in Literature Cited. If such a paper would be useful in the evaluation of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited. Personal communications and unpublished experiments should be treated as footnotes to the text.

Numbers. Use Arabic numerals throughout, including those in tables and figures. If possible, avoid beginning a sentence with a numeral; when necessary, numbers so used should be spelled out as well as any units immediately *following*.

Abbreviations and symbols. Letters in abbreviations such as DPN or IU are not spaced and periods are omitted except when the abbreviation might be read as another word. Following is a list of the more common abbreviations and symbols used in the *Journal*:

average	avg (*in tables*)
centimeter(s)	cm
counts per minute	count/min
cubic centimeter(s)	cm^3
cubic millimeter	mm^3
degree(s)	°
degrees of freedom	df (*in tables*)
gram(s)	g
international unit(s)	IU (*to be used only when weight can not be given*)
kilogram(s)	kg
liter(s)	(spell out)
meter(s)	m
microgram(s)	μg (not γ)
micromicrogram(s)	$\mu\mu$g
microcurie(s)	μCi
micron(s)	μ
micromicron(s)	$\mu\mu$
micromolar (unit of concn)	μM
micromole (unit of mass)	μmole
milligram(s)	mg
milligrams %	(*never use*)
milliliter(s)	ml
millimeter(s)	mm
millimicrogram(s)	mμg
millimicron(s)	mμ
millimole(s)	mmole
molar (mole per liter)	M
parts per million	ppm
per cent	%
probability (in statistics)	*P*
square centimeter	cm^2

[1] Style Manual for Biological Journals 1960 American Institute of Biological Sciences, 2000 P street, N. W., Washington 6, D. C.

square meter	m^2
square millimeter	mm^2
standard deviation	SD
standard error	SE
t (Fisher's test)	*t*
weight (in tables)	wt

Other commonly accepted abbreviations may be found in the *Style Manual for Biological Journals*.[2] The isotope designation of a labeled compound should ordinarily appear *before* the name of the compound to which it applies. When following a symbol for a compound, it should be written as superscript (as, ^{14}C); when the name of the compound is spelled out, the isotope designation should be written on the same line (as, carbon-14).

Tables and figures. Follow form in current issues for the use of upper and lower case letters and italics. Authors are urged to economize on space used for tables and figures. These should fit one column width (2⅝ inches) or when necessary, two column widths (5½ inches). A charge will be made by the publisher for that space used for tables and figures which exceeds one-quarter of the space used for the manuscript exclusive of tables and figures. A table or figure should be constructed to be intelligible without reference to the text. Lengthy tabulation of essentially similar data can often be avoided by giving the number of experimental results and their mean values, with standard deviations or ranges within which the values fall. Statements that significant differences exist between the mean values of two groups of data should be accompanied by indications of probability derived from the test of significance applied. Units of measure should be indicated clearly two spaces above the first value in a column.

Original drawings, with two reproductions, to be sent to reviewers, or in the case of photographs, the original and two glossy prints, should accompany the manuscript. They should be marked on the back in ink with the author's name, complete address, and with the figure numbers. Such drawings and photographs must not exceed 8½ × 11 inches in size and must be at least 5¼ inches wide in order to fit the 2⅝-inch single column width when reduced by one-half. When a complicated figure requires more space for clarity, a proportionately larger illustration will be acceptable. But two copies of *prints* should be submitted on sheets of the same size as the text. Or if prints are smaller, they should be affixed to sheets of manuscript size. Oversize or undersize figures are difficult to handle in editing. Legends (including any keys to symbols or charts) should appear on a separate sheet. Drawings should be on white or blue-white paper or bristol board — not cream-white. They should be prepared for reproduction as line or halftone engravings. Letters and numbers should be uniform and large enough so that no character will be less than 2 mm high after reduction. A line 0.4 mm wide reproduces satisfactorily when reduced by one-half.

Figures should be lettered (preferably by stencil) in *black* India ink. For any charts made on cross section paper, use India ink on paper printed in light blue only. Charts drawn in India ink should be so executed throughout, with no typewritten material included. Graphs and charts should be given consecutive figure numbers as they will appear in the text.

Page charge. For manuscripts received on and after December 1, 1962, a charge of $20.00 per printed page in the Journal of Nutrition will be made by the American Institute of Nutrition to authors or institutions whose research funds permit charges for publication. Authors will be notified of the cost after they receive the page proofs, and will have the opportunity at that time to signify whether the research reported was supported by the type of funds that permit such charge for publication. Ability to pay this charge will *in no way* prejudice the acceptance of a manuscript. Billing will be handled directly by AIN; the Editor's Office will not see these charges, nor be advised concerning the author's payment or nonpayment of the page charge. A separate standard charge is made by the publisher for reprints, excess tabular material and photoengraving, and for changes in proof that are considered excessive or unnecessary.

Action to institute a page charge for publication in the Journal of Nutrition was taken at the April 1962 meeting of the AIN. Proceedings of this meeting are printed in

[2] See footnote 1.

the Journal of Nutrition, 78: 120–132, 1962.

Reprints. Reprint order forms and rates will be sent with page proofs. Anyone other than an author desiring reprints must have orders approved by the author, and give written assurance that (1) distribution will be restricted to those interested in the science of nutrition, and (2) no advertising or imprinting will be attached to the reprints thus furnished.

American Documentation Institute. To conserve space in the Journal, lengthy descriptions of experimental procedures, detailed data such as that on individual animals or subjects, extra figures and other material considered important supplementary information, may be deposited with the American Documentation Institute, Library of Congress, through the Editor's Office with a footnote reference.

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