Methionine-responsive Liver Damage in Young Pigs Fed a Diet Low in Protein and Vitamin E [1]

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ABSTRACT A combined protein-vitamin E deficiency syndrome causing severe liver damage in the pig was studied by manipulating dietary levels of methionine, choline and selenium. Yorkshire pigs weaned at 3 weeks of age, weighing an average of 5 kg, were used in 4 experiments. During the 8-week experimental period the animals were fed a basal diet containing 3% isolated soy protein and 25% corn oil; it was marginal in vitamin E and selenium but adequate in all other nutrients. Consumption of this diet resulted in the development of liver necrosis which was most obvious histologically at weeks 2 and 4; at week 8 severe postnecrotic scarring of the liver predominated with little evidence of acute necrosis. The liver damage was completely preventable by supplementation with α-tocopherol, or selenium, or both. Choline supplementation aggravated the liver damage; however, methionine supplementation provided considerable, and in some cases, complete protection against necrosis and scarring but did not prevent the appearance of hyaline bodies in the hepatocytes. The methionine effect was not related to contamination of the supplemental methionine with selenium.

Previous reports from this laboratory have described the biochemical, morphologic and behavioral changes observed in weanling pigs fed experimental diets low in protein, but adequate in all other known nutrients (1, 2). The objective was to develop in the pig an experimental model which mimicked severe protein-calorie malnutrition in the child. Such a system offers many possible avenues of study; two which have been explored are the effect of the experimental protein deficiency on learning behavior (3), and the relative efficiency of several different dietary proteins in rehabilitating the severely protein-calorie malnourished pig (4).

The advantages of using animals, particularly the pig, in experimental model systems have been discussed elsewhere (5); one such advantage is that, in animal studies, the complicating effect of nutrient deficiencies other than protein can be eliminated so that only the protein deficiency effect is seen This may also be considered a disadvantage, however, since seldom, if ever, does protein deficiency occur unaccompanied by other nutrient deficiencies in the human situation. Thus, when the observation was made that lowering the vitamin E content of the experimental low protein diet to submarginal levels resulted in liver damage of a type not encountered in simple protein deficiency, it was decided to study the combined protein-vitamin E deficiency more closely.

The present studies were designed to evaluate the effect of dietary manipulation of nutrients, particularly methionine, choline and selenium on the protein-vitamin E deficiency syndrome, using liver damage as the principal criterion.

EXPERIMENTAL

General. Seventy-four Yorkshire pigs weaned at 21 days of age and averaging approximately 5 kg of body weight were used in 4 experiments. The animals were assigned at random by litter, weight and sex to dietary treatments. The animals were housed in pairs in concrete-floor pens, 2 m × 1 m with plywood partitions. Water was supplied ad libitum from cast-iron troughs and the experimental diets were supplied ad libitum from metal self-feeders.

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The basal diet used in all experiments was a 3% soy protein diet with a selenium level of 0.018 mg/kg diet and an α-tocopheryl acetate level of 7.60 IU/kg diet. The composition of the basal diet is shown in table 1. Room temperature was maintained near 22° with a thermostatically controlled electric space heater.

Body weight and feed intake were recorded weekly. Blood samples were drawn from the anterior vena cava into a heparinized syringe and placed immediately into test tubes in a container with cracked ice. Total plasma proteins were determined by the method of Gornall et al. (6) and electrophoretic patterns were run on the same samples using the Spinco Model R electrophoresis system. With the exception of experiment 1, all animals were killed on day 56 either by electrocution (exps. 1 and 4) or sodium pentobarbital. At necropsy the liver was weighed fresh on removal from the body; after removal of tissue for histopathology, the liver was homogenized and frozen. Liver lipid was subsequently determined by ether extraction of the homogenized, freeze-dried sample. Tissues selected for histopathology were routinely fixed in Bouin's solution. In addition, liver was fixed in formalin for fat-staining and in alcohol for glycogen-staining. Paraffin-embedded tissues were sectioned at 6 μ; frozen tissues at 10 μ. All tissues were stained with hematoxylin-eosin; in addition, liver sections were stained with Sudan III for lipid and alcohol-fixed liver with Best's carmine for glycogen. The severity of liver damage after 56 days on experiment was evaluated on the basis of 6 criteria: the presence of triad fibrosis, hemosiderosis, bile duct proliferation, eosinophilic leukocytosis, interlobular fibrosis and pseudolobulation. Each criterion was scored on a scale ranging from zero for absence of change to 5 for maximal severity. Thus, with 6 criteria, each scored zero to 5, the maximal severity score was 30.

Experiment 1. Preliminary experiments not reported here had indicated that pigs subjected to a low protein diet with a marginal vitamin E content showed severe liver damage when killed after 8 weeks. The following serial experiment was designed to evaluate the pathogenesis of the liver lesions.

TABLE 1

Composition of basal and control diets

	Basal	Control
	%	%
Glucose [1]	40.6	13.6
Dextrin [2]	25.0	25.0
Corn oil [3]	25.0	25.0
Mineral mix [4]	5.4	5.4
Vitamin mix [5]	1.0	1.0
Isolated soybean protein [6]	3.0	30.0
Vitamin E [7]	—	+
Selenium [8]	—	+

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Nutritional Biochemicals Corporation, Cleveland.
[3] Mazola, Corn Products Company, Argo, Illinois.
[4] Mineral mix contributed the following levels per kg of diet: (in g) $CaHPO_4 \cdot 2H_2O$, 15.4; $CaCO_3$, 12.30; KH_2PO_4, 17.2; NaCl, 6.1; $CuSO_4 \cdot 5H_2O$, 0.57; $FeSO_4 \cdot 7H_2O$, 0.66; $MnSO_4 \cdot H_2O$, 0.185; $ZnCO_3$, 0.66; MgO, 0.82; $CoCl_2 \cdot 6H_2O$, 0.011; and KI, 0.0004.
[5] Vitamin mix contributed the following levels per kg of diet: (in IU) vitamin A, 5720; vitamin D_3, 660; vitamin E, 7.60; (in mg) thiamine·HCl, 3.96; riboflavin, 9.90; niacin, 66.00; Ca pantothenate, 39.60; choline dihydrogen citrate, 3960.00 (in exp. 3), 6,458.00 (in exps. 1, 2 and 4); vitamin B_{12}. 0.066 (in exp. 3), 0.198 (in exps. 1, 2 and 4); inositol, 220.00; folic acid, 2.20; menadione, 4.40; pyridoxine, 3.30; biotin, 0.33 (in exp. 3), 1.01 (in exps. 1, 2 and 4).
[6] Promine, Central Soya, Chicago.
[7] Supplemented as α-tocopheryl acetate to supply 100 IU of vitamin E activity per kilogram of diet.
[8] Na_2SeO_3 to supply 0.5 mg selenium per kilogram of diet.

Sixteen Yorkshire pigs weighing an average of 4.5 kg were assigned to 4 treatments, 4 animals/treatment. All animals were assigned to the basal 3% soy protein diet (table 1). Four animals were killed every 2 weeks for an 8-week period. A terminal blood sample was obtained from each pig immediately before it was killed for determination of plasma protein levels. At necropsy, sections were taken from liver, kidney, pancreas, spleen, lung, esophagus, trachea, stomach, small intestine, large intestine, thyroid, adrenal, skeletal muscle and cardiac muscle.

Experiment 2. Eighteen pigs were assigned to 4 experimental diets, 4 pigs/diet; 2 animals were assigned to a control diet. The basal diet was fed alone, supplemented with 0.5 ppm selenium (supplied as sodium selenite), supplemented with α-tocopheryl acetate to a level of 100 IU of vitamin E activity per kilogram of diet and supplemented with both selenium and vitamin E at the indicated levels. The control diet was a 30% soy protein diet supplemented with vitamin E and selenium

(table 1). All diets were fed for a period of 56 days at which time blood samples were drawn and the animals killed. Only liver was taken for histopathology.

Experiment 3. Sixteen Yorkshire pigs were assigned at 3 experimental diets and to a control diet. The basal diet was fed alone, supplemented with 0.10% DL-methionine, and supplemented with 0.10% choline supplied as choline dihydrogen citrate. The control diet was a 30% soy protein diet (table 1). The animals were bled and killed on day 56. Liver was obtained on necropsy and processed for histopathology. The supplemental methionine used was 98% DL-methionine [3] with a determined selenium content of 0.018 ppm.

Experiment 4. Although the results of experiment 3 showed that supplementation of the basal diet with 0.10% DL-methionine completely prevented liver damage, an experiment was designed to determine whether the observed effect with supplemental methionine was due to contamination of methionine with selenium or whether the effect was a true amino acid effect. Twenty-four pigs were assigned to 5 experimental diets and to a control diet. The basal diet was fed alone, supplemented with 0.10% DL-methionine, supplemented with α-tocopheryl acetate to a level of 100 IU of vitamin E activity per kilogram of diet, supplemented with 0.5 ppm selenium (as sodium selenite), and supplemented with seleno-DL-methionine (0.00447 mg/100 kg diet); this amount of selenomethionine supplies 0.000018 ppm selenium which is equal to the amount of selenium supplied by the addition of 0.10% DL-methionine to the basal diet. Blood samples for plasma protein determinations were taken at weeks 2, 4, 6 and 8. Serum glutamic-oxaloacetic transaminase (SGOT) activity was measured at weeks 2, 4, 6 and 8 using the Sigma method.[4] For histopathology, the same protocol was followed as described in experiment 1. In addition, selected tissues were stained with Ladewig's modification of Mallory's connective tissue stain, Weigert's elastin stain and phloxine.

RESULTS

Experiment 1. The development of the fatty liver as judged chemically is shown in figure 1. Not until week 6 of the deficiency do hepatic lipid levels rise significantly above normal. Between weeks six and eight hepatic lipid levels essentially double. There is, however, considerable variation in lipid levels at week eight as evidenced by the large standard error of the mean.

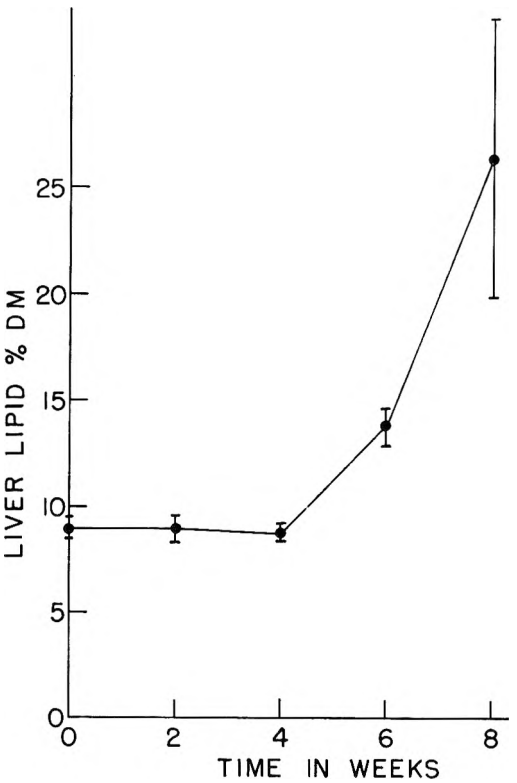

Fig. 1 Fatty liver development during the 8-week protein-depletion period. (Lipid expressed as % liver dry matter.)

The results of the pathologic examination of the liver at the biweekly intervals are described below. There was no mortality in any of the treatments.

Two weeks. Fatty metamorphosis was not demonstrated histologically. Glycogen was moderately increased in the cord cells.

In 2 of the 4 pigs there were regressive liver changes. They were focal and rather mild. Lesions included hyalinization of the cytoplasm with the spherical, acidophilic hyaline bodies typically surrounded by an

[3] Feed grade, Dow Chemical Company, Midland, Michigan.

[4] Sigma Technical Bull. 505 (1964), Sigma Chemical Company, St. Louis.

empty halo, and pyknosis, rhexis and even lysis of the cord cell nuclei. Reparative processes were not observed.

Four weeks. The liver cord cells showed no fatty metamorphosis. Glycogen in nonnecrotic cells was still more increased.

Two of the pigs showed focal necrosis throughout the liver. Reactive changes consisted of mononuclear cell infiltration, hemosiderosis of reticuloendothelial cells and a mild fibroplasia. In the remaining 2 pigs necrosis was extensive and reparative processes more advanced.

Six weeks. There was a mild sudanophilic reaction in the periphery of the lobules. Glycogen was very prominent in the cord cells.

Although focal acute necrosis was observed in some instances, the predominant changes in the liver were chronic proliferative. The lesions were those listed under Experimental and they were of mild-to-moderate degree.

Eight weeks. Fatty metamorphosis was advanced throughout the lobules and glycogen staining reaction was intense.

Two of the pigs exhibited mild-to-moderate chronic liver changes, whereas the lesions were severe in the other 2 pigs.

The results of this serial experiment indicate that young pigs fed a diet low in protein and vitamin E develop hepatic necrosis of varying severity — massive, submassive or focal — but only rarely of sufficient acuity to precipitate death. In surviving animals, the response of the liver to the necrotic insult was a chronic scarring (fig. 2), the severity dependent upon the extent of the previous necrosis. Massive or submassive necrosis resulted in

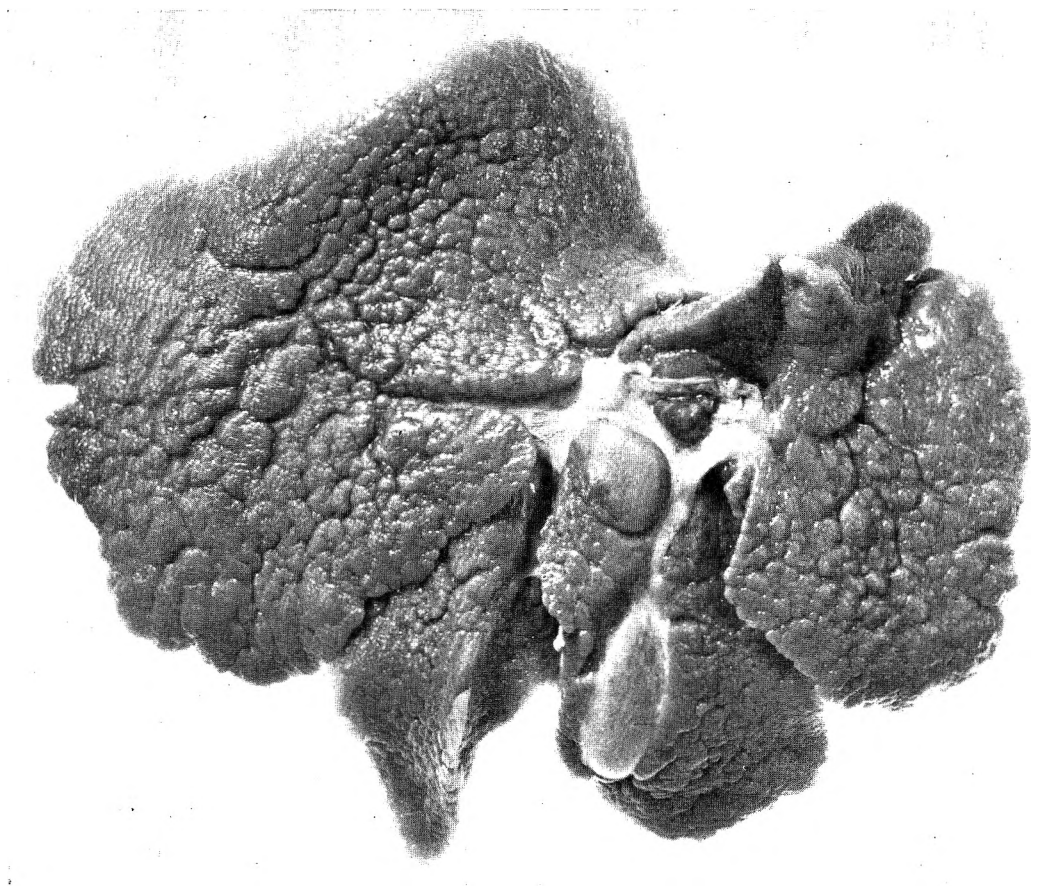

Fig. 2 Extensive postnecrotic scarring in liver of animal on basal diet for 8 weeks.

a collapse of large areas of liver parenchyma with infiltration of fibrous tissue and chronic inflammatory cells and with a proliferation of bile ductules. Focal necrosis resulted in a much less widespread fibrosis characterized by monolobular and pseudolobular nodules.

Experiment 2. The results on weight change and liver damage (table 2) show that supplementation of the basal diet with selenium and vitamin E, either together or separately, completely prevented weight loss and liver damage. Pigs receiving the basal diet lost weight and suffered a 100% incidence of liver damage with an average severity score of 14.5. None of the animals receiving the supplemented diets developed liver damage and all gained a small amount of weight. Selenium or vitamin E alone was as effective in preventing liver damage as when fed together. It should be pointed out that a severity score of zero does not necessarily denote a normal liver, only the absence of any of the 6 criteria enumerated in the description of experimental procedure. The control animals had normal livers, but the livers of the 3 supplemented basal groups all showed the characteristic periportal fatty liver of protein deficiency. Selenium or vitamin E supplementation had no effect on liver lipid levels in the protein-deficient groups (table 3). The liver lipid levels of protein-deficient animals show considerable variation but are consistently higher than control values.

There was no statistically significant effect of either selenium or vitamin E supplementation on serum protein levels (table 3). All of the serum protein parameters of the control groups were significantly greater ($P < 0.01$) than those of the protein-deficient groups. In the supplemented basal groups, both the albumin-to-globulin ratio and albumin expressed as a percentage of total serum protein tended to increase with supplementation of selenium, or vitamin E, and particularly with

TABLE 2

Effect of selenium and vitamin E supplementation on experimental liver damage in young pigs (exp. 2)

Diet no.	Diet designation	Wt change	Liver damage	
			Incidence	Severity score (0–30)
		g	%	
1	Basal diet	−137 [1]	100	14.5
2	+ Se, 0.5 ppm	+300	0	0
3	+ Vitamin E, 92.4 IU	+367	0	0
4	+ Se, 0.5 ppm + vitamin E, 92.4 IU	+263	0	0
5	Control diet	+19,775	0	0

[1] Weight change between day zero and day 56.

TABLE 3

Effect of selenium and vitamin E on liver lipid and serum protein levels (exp. 2)

Diet no.	1	2	3	4	5
Diet designation	Basal	Basal + Se	Basal + vitamin E	Basal + Se + vitamin E	Control
Liver lipid (dry basis), %	19.44	26.56	18.50	16.76	7.76 **
Serum proteins					
Total, g/100 ml	3.68	3.60	3.22	3.18	6.55 **
Albumin, g/100 ml	1.28	1.32	1.20	1.28	3.50 **
Globulin, g/100 ml	2.38	2.28	2.02	1.90	3.05 **
Albumin:globulin	0.54	0.58	0.62	0.72	1.15 **
Albumin, % of total	35.32	36.48	37.12	40.98	53.50 **

** Significantly different from basal diets ($P < 0.01$).

both. This effect, however, rather than being a consequence of increasing serum albumin levels with supplementation, was due to decreasing serum globulin levels in diets 1 through 4.

The results of this, and the previous experiment, clearly indicate that the liver damage observed was a manifestation of vitamin E-selenium deficiency.

Experiment 3. Supplementation of the basal diet with 0.10% DL-methionine completely prevented liver damage (table 4). Methionine supplementation, however, did not prevent the appearance of numerous large hyaline bodies within the liver cells. Choline supplementation had no protective effect; rather, it appeared to aggravate the liver damage. Both choline and methionine supplementation had a significant ($P < 0.05$) lipotropic effect (table 5) reducing liver lipid levels in diets 2 and 3 by 50% under diet 1 levels, but not back to control levels. The serum protein parameters showed little of significance (table 5) with the possible exception of the somewhat higher serum albumin level of the methionine group compared with the basal or basal plus choline groups. This may reflect an improvement in liver function consequent to the lack of liver damage in the methionine-supplemented group.

This experiment was repeated with similar results. Methionine and choline again had a lipotropic effect on liver lipid levels; the preventive action of methionine on liver damage was confirmed, as was the aggravating effect of choline which, in the repeated experiment, was more pronounced than in the original experiment. The question as to the modus operandi of the preventive effect of methionine remained unanswered. The methionine source used was a feed grade containing not less than 98% DL-methionine; by analysis this methionine source was found to contain 0.018 ppm selenium. The possibility existed that the preventive effect of methionine was a selenium-contaminant effect. It seemed inconceivable that the infinitesimal (0.000018 ppm) amount

TABLE 4

Effect of methionine and choline supplementation on experimental liver damage in young pigs (exp. 3)

Diet no.	Diet designation	Wt change	Liver damage	
			Incidence	Severity score (0–30)
		g	%	
1	Basal diet	+50 [1]	100	9.00
2	+ 0.10% DL-methionine	+475	0	0.00
3	+ 0.10% choline	−250	100	14.30
4	Control diet	+16,800	0	0.00

[1] Weight change between day zero and day 56.

TABLE 5

Effect of methionine and choline supplementation on liver lipid and serum protein levels (exp. 3)

Diet no.	1	2	3	4
Diet designation	Basal	Basal + 0.10% DL-methionine	Basal + 0.10% choline	Control
Liver lipid (dry basis), %	38.29	19.15	17.99	10.02
Serum proteins:				
Total, g/100 ml	3.88	4.43	4.23	6.27
Albumin, g/100 ml	1.35	1.62	1.33	3.33
Globulin, g/100 ml	2.53	2.81	2.90	2.94
Albumin:globulin	0.53	0.58	0.46	1.13
Albumin, % of total	35.00	36.15	31.00	52.67

of selenium supplied by the addition of 0.10% DL-methionine to the basal diet could have prevented the liver damage. Nevertheless, this possibility was investigated in the last experiment by matching the selenium supplied as a contaminant of methionine with an equal amount of selenium supplied in the form of seleno-DL-methionine.[5] In this way it was hoped to separate the methionine (i.e., amino acid) effect and the selenium-contaminant effect.

Experiment 4. Supplementation of the basal diet with 0.10% DL-methionine afforded almost complete protection against liver damage (table 6); however, supplementation with seleno-DL-methionine to supply the same amount of selenium as supplied by 0.10% of DL-methionine had no protective action against liver damage. Supplementation with vitamin E or selenium protected completely against liver damage, confirming the results of experiment 2. The liver lipid and serum protein data showed nothing that had not been observed in previous experiments and that is not reported here. Of interest are the data on change of SGOT levels with time (table 7). At week 2, the basal diet had a SGOT level significantly elevated ($P < 0.05$) above the control value. The methionine- and seleno-DL-methionine-supplemented groups both had somewhat elevated SGOT levels, but neither were significantly different from control or basal values. By week 4, all SGOT levels had returned to control values and remained there throughout the experiment. The elevations in SGOT activities were not striking, but did occur in those groups where evidence of liver damage was found on necropsy.

The results indicate that the protective action of DL-methionine on liver damage is not a consequence of selenium contamination but rather is a true amino acid effect.

[5] Grade 1, containing 40.26% selenium, Cyclo Chemical Company, Los Angeles.

TABLE 6

Failure of seleno-DL-methionine to prevent experimental liver damage in young pigs (exp. 4)

Diet no.	Diet designation	Wt change	Liver damage	
			Incidence	Severity score (0–30)
		g	%	
1	Basal diet	−350 [1]	100	12.50
2	+ 0.10% DL-methionine	+188	50	2.75
3	+ Vitamin E, 92.4 IU	−237	0	0.0
4	+ Se, 0.5 ppm	+62	0	0.0
5	+ Seleno-DL-methionine [2]	−125	100	13.50
6	Control diet	+10,000	0	0.00

[1] Weight change between day zero and day 56.
[2] Supplies 0.000018 ppm Se.

TABLE 7

Change in serum glutamic-oxaloacetic transaminase with time (exp. 4) [1]

Diet no.	Diet designation	SGOT			
		2 weeks	4 weeks	6 weeks	8 weeks
		Sigma-Frankel units			
1	Basal diet	87.5 [a]	24.7	17.5	16.0
2	+ 0.10% DL-methionine	43.5 [ab]	22.2	21.2	21.2
3	+ Vitamin E, 92.4 IU	20.8 [b]	15.2	14.5	12.0
4	+ Se, 0.5 ppm	18.0 [b]	16.8	16.7	16.5
5	+ Seleno-DL-methionine	61.0 [ab]	21.2	23.5	19.5
6	Control diet	21.5 [b]	12.8	11.5	11.5

[1] Means within the same week having the same superscript are not significantly different ($P < 0.05$); no significant differences in weeks 4, 6 or 8.

DISCUSSION

Vitamin E-selenium-responsive diseases have been described in several species and include dietary liver necrosis in the rat (7) and hepatosis diaetetica in the pig (8). Three separate dietary factors — vitamin E, selenium and cystine — all had been reported to protect against dietary liver necrosis in either rats or pigs. It became obvious in the late 1950's (9) that commercial L-cystine was usually contaminated by 1 to 2 μg of selenium per gram of amino acid. Many of the experiments reporting a protective effect of sulfur amino acids in dietary liver necrosis came under question. Careful experimentation using sulfur amino acids free of selenium contamination, however, showed that sulfur amino acids, although not affording complete protection, delayed the onset of dietary necrotic liver degeneration in the rat. This effect was mediated by a potent sparing action of the sulfur amino acids on the requirement for vitamin E (7).

The results presented in this paper are entirely compatible with this concept. Vitamin E-selenium-responsive liver damage was completely prevented by vitamin E or selenium supplementation, but only partially protected by methionine. In experiment 4, methionine supplementation afforded considerable protection but did not prevent the development of mild fibrosis in the livers of 2 animals. In experiment 3, there was no evidence of fibrosis in the methionine-treated group, but cytoplasmic hyaline bodies were abundant.

Hyaline bodies (alcoholic hyaline, Mallory bodies) have received much attention as a feature of liver injury (10). Hyaline bodies were originally described by Mallory (11) as pathognomonic of alcoholic liver cirrhosis, but similar eosinophilic cytoplasmic masses have been observed in nonalcoholic cirrhosis (12), in noncirrhotic liver, and in certain types of experimentally damaged animal liver (13–15). The ultrastructural interpretations of the various forms of cytoplasmic hyaline in man and animals are summarized by Steiner et al. (16). Two types of cytoplasmic alterations, namely, swelling and clumping of the mitochondria and disruption of the endoplasmic reticulum, are thought to result in the appearance of hyaline bodies. Svoboda and Higginson (17) have shown that the earliest lesion in the liver of rats fed a necrogenic diet low in α-tocopherol and cystine was a swelling of the mitochondria. Todd and Krook (15) have reported that the earliest histological change in vitamin E-selenium deficiency in the bovine was the appearance of cytoplasmic hyaline bodies. The demonstration of hyaline bodies in the liver of methionine-treated animals may therefore be interpreted as evidence of cellular injury. The protective effect of methionine, while considerable, was nevertheless only partial and possibly mediated by sparing vitamin E. The results of experiment 4 indicate that selenium contamination was not a significant factor in the protective action of methionine.

It remains to be established definitely whether methionine or one of its oxidation products is the protective agent in dietary liver necrosis. Schwarz (7) has proposed that, since homocysteine and methionine are as effective as cystine, and the conversion of cystathionine to cysteine is irreversible in mammals, the protective effect appears to originate at the cystine-cysteine level or below. Hathcock and Scott (18) have recently shown that cysteine, not methionine, is the metabolically active sulfur amino acid in the prevention of nutritional muscular dystrophy in vitamin E-deficient chicks.

Choline supplementation in experiment 3 considerably increased the severity of liver damage. Mackenzie and duVigneaud (19) have shown that availability of methyl groups, such as supplied by choline, tends to keep sulfur amino acids in the methionine area, whereas lack of methyl groups increases the rate of catabolism of sulfur amino acids. Thus, choline supplementation may reduce the conversion of methionine to cysteine, thereby increasing the severity of liver damage. The same reasoning explains why methionine supplementation in experiment 4 (choline level, 0.26%) did not completely prevent the appearance of fibrosis, whereas methionine supplementation in experiment 3 (choline level, 0.16%) did.

The finding of postnecrotic hepatic scarring in protein-vitamin E-deficient pigs may have relevance to the continuing con-

troversy about liver cirrhosis as a possible sequel to protein malnutrition in the human. As Schwarz (20) remarks, the term "protein malnutrition" may not properly define the situation from a strictly biochemical point of view. In addition to amino acids, proteins usually also carry a great many vitamins and trace elements either chemically bound or as contaminants. The word "protein," while of great value in the description of the nutritional status of a population, is of little value when it comes to the search for specific etiological mechanisms in biochemical terms. The evidence for vitamin E deficiency in human protein malnutrition is considerable and accumulating (21–23). Little attention has been directed toward selenium in protein malnutrition, but existing evidence points to the possibility of selenium deficiency in kwashiorkor (24, 25).

Waterlow and Bras (26, 27) have advanced a dual theory of the etiology of cirrhosis which postulates that toxic, infective or parasitic agents, when combined with malnutrition, may play a causative role. Our evidence shows that protein malnutrition in the pig, when associated with vitamin E-selenium deficiency, results in severe postnecrotic scarring of the liver. The possibility of a similar deficiency precipitating liver cirrhosis in the protein-malnourished human should be investigated.

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Effect of Dietary Lipid upon Some Enzymes of Significance in Biogenic Amine Metabolism in the Rat [1]

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ABSTRACT Activities of certain enzymes concerned with biogenic amine metabolism were influenced significantly by the dietary lipid. Monoamine oxidase, 5-hydroxytryptophan decarboxylation, and in vitro metabolism of 5-hydroxytryptophan to end products were highest in livers from rats fed 7% levels of cod liver oil, linseed oil, corn oil or a combination of linseed and cod liver oils, in comparison with similar data from animals fed beef fat or low levels of corn oil. No differences were found in brain enzyme activity among the experimental groups. Fatty acids from liver phospholipids reflected the differences due to the dietary lipid, but no significant differences were found in liver lipid content, or in liver and brain nitrogen, RNA, and DNA levels.

Studies from our laboratory (1–3) as well as by other investigators (4–7) have shown that the fatty acid composition of lipids, even phospholipids from cellular and subcellular structures, can readily be altered by feeding lipids of different compositions. One may, therefore, reasonably ask whether enzymes, responses to drugs, and other cellular functions might differ as a result of these compositional changes in lipoprotein structures obtained by feeding various lipids. Although a plethora of information has accumulated concerning the effects of dietary lipids upon atherogenesis, cholesterol, lipid levels and lipid metabolism, less work has been described concerning the effects of dietary lipids upon other biological variables. Previous reports from this laboratory have shown that swelling responses of liver mitochondria (8), in vitro depression of oxidative phosphorylation by chlorpromazine (9), and in vivo depression of ^{32}P labeling of rat brain phospholipids by chlorpromazine [2] were significantly affected by feeding different fats and oils. Tepperman and Tepperman (10) found increased activities in malic enzyme, hexose monophosphate dehydrogenase, and NADPH oxidase in livers from rats fed saturated fat diets over relatively short periods. Erythrocytes from rats fed coconut oil were more easily hemolyzed by glycerol and thiourea than cells from rats fed corn oil (11). A number of reports have described the effects of fasting (12–13) and of protein deprivation (14–17) on various enzymes and animal behavior. The present investigation is concerned with the effects of feeding nutritionally adequate diets that furnish different lipids upon some enzymes known to be prominent in biogenic amine metabolism.

EXPERIMENTAL

Weanling male albino rats, of the Sprague-Dawley strain, weighing 40 to 45 g, were fed semisynthetic diets (tables 1, 2) similar to those previously described (18). These diets are adequate in the known nutrients and are isocaloric with respect to the protein, vitamin, and mineral contents. Animals were killed during the times indicated on the tables and were of comparable weights. Generally, the largest rats of each group were killed earlier, to obtain as much uniformity as possible. Liver and brain homogenates were prepared in ice-cold 0.25 M sucrose in concentrations suitable for various assays. All incubations were made in a water-bath

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TABLE 1

Experimental diets

	A				B		
	Corn oil percentages				Lipid percentages		
	10	2	1	0.5	7	0.8	0.5
	parts	*parts*	*parts*	*parts*	*parts*	*parts*	*parts*
Casein (vitamin test)	25	25	25	25	25	25	25
Salt mixture 446 [1]	4	4	4	4	4	4	4
Dextrose, U.S.P.	61	78.6	81.0	82.25	64	78.04	78.76
Lipid [2]	10	2.2	1.1	0.55	7	0.86	0.54
Total	100	109.8	111.1	111.8	100	107.90	108.30
Choline H_2 citrate	0.33	0.33	0.33	0.33	0.33	0.33	0.33
Vitamin mix [3]	0.33	0.33	0.33	0.33	0.33	0.33	0.33

[1] Salt mixture described by Mameesh and Johnson (19), modified to furnish 0.38 ppm of chromium and 0.16 ppm of selenium as sodium selenite to the diet.

[2] *d*-α-Tocopheryl acetate (furnished by Distillation Products Industries, Rochester, New York) was added to the lipids to supplement 400 μg/g of dietary lipid in the higher lipid containing diets, and 2.0 mg/g of lipid in the diets containing lower levels of corn oil.

[3] Vitamin mixture furnishes the following levels in the diet (mg/kg or the isocaloric equivalent): *i*-inositol (*meso*), 111; *p*-aminobenzoic acid, 111; menadione, 50; niacin, 100; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca-D-pantothenate, 67; folic acid, 2; biotin, 0.45; and vitamin B_{12}, 0.03. Vitamins A (2500 IU) and D (180 IU) were given orally in 2 drops of oleum percormorphum per week. Animals fed diets containing cod liver oil were given 1 drop/week.

shaker at 37°. Specific inhibitors [3] were added in concentrations described in the results. Decarboxylation of 5-hydroxytryptophan (5-HTPD) was determined by the method of Snyder and Axelrod (20) using side-chain labeled 5-HTP-2-^{14}C as the substrate. In all instances of ^{14}C counting, aliquots were taken for assay by liquid scintillation counting using Bray scintillation fluid (21). Monoamine oxidase (MAO) determinations were made using the radioactive tryptamine procedure of Wurtman and Axelrod (22), or the fluorometric method of Kraml (23) with kynuramine as the substrate. In the latter case, 0.6 M perchloric acid instead of 10% trichloroacetic acid was used to stop the reactions. Catechol-O-methyltransferase (COMT) was determined by the method of McCaman (24). Metabolism in vitro of 5-hydroxytryptophan (5-HTP) was assayed by the method of Feldstein and Wong (25). Radioactive serotonin (5-HT) and 5-HTP-^{14}C were separated and determined as described; but in a modification of the procedure, aliquots of the ethyl ether extract were assayed for ^{14}C before and after the extraction step using NaCl saturated 0.5 M borate buffer (pH 10). This difference represented 5-hydroxyindoleacetic acid (5-HIAA) and the final ether fraction contained 5-hydroxyindoleacetaldehyde plus 5-hydroxytryptophol (neutrals). In all the enzyme determinations, control flasks which included the acid or buffer used to stop the reactions were incubated with the experimental samples. Liver nitrogen was determined by a Nessler procedure (26), liver lipids by the gravimetric procedure of Sperry and Brand (27–28), and RNA and DNA by the method of Monro and Fleck (29), using the diphenylamine reaction for the final determination of DNA. Fatty acid compositions of liver phospholipids and brain lipids were determined as previously described (3).

RESULTS

Unsaturated fatty acid compositions of liver phospholipids and brain lipids shown in table 2 illustrate the differences found in the experimental groups. Liver phospholipids from rats fed 7% corn oil contained high proportions of ω6 essential fatty acids (EFA) with 4 and 5 double bonds (30). These totaled about 22%, as compared with 8% in animals given 7% beef fat. Feeding diets containing linseed or cod liver oils, which supplied high levels of ω3 polyunsaturated fatty acids (PUFA) (30),

[3] Compounds used were iproniazid, Hoffmann-La Roche, Inc., Nutley, N. J.; β-phenylisopropylhydrazine hydrochloride (JB-516, Catron), Lakeside Laboratories, Milwaukee, Wisc.; *N'*-methyl-*N'*-(3-hydroxybenzyl)-hydrozinium dihydrogen phosphate (NSD-1034) and 4-bromo-3-hydroxybenzyloxyamine trihydrogen phosphate (NSD-1055), Smith & Nephew Research, Ltd., Ware, Herts, England; and α-methyl-dihydroxyphenylalanine (α-methyl-DOPA), Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 2

Percentages of unsaturated fatty acids in liver phospholipids from rats fed various dietary lipids [1]

Fatty acid	Dietary lipid								
	7% Beef fat	0.3% Corn oil + 6.7% beef fat	0.5% Corn oil	0.8% Corn oil	2% Linseed oil + 5% cod liver oil	7% Linseed oil	7% Cod liver oil	7% Corn oil
	(5) [2]	(8)	(4)	(5)	(6)	(5)	(5)	(8)
16:1 (Palmitoleic)	3.4 ± 0.4 [3]	2.5 ± 0.2	4.3 ± 0.1	4.5 ± 0.6	4.1 ± 0.5	2.6 ± 0.4	5.2 ± 0.7	1.7 ± 0.2
18:1 (Oleic)	20.3 ± 1.3	17.6 ± 0.3	16.0 ± 0.7	16.4 ± 1.3	16.6 ± 1.3	13.8 ± 1.6	17.8 ± 2.1	10.0 ± 0.5
20:3 (Eicosatrienoic–ω9)	7.3 ± 0.4	2.8 ± 0.2	1.9 ± 0.5	1.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.05	0.1 ± 0.02	0.1 ± 0.05
Essential (ω6)								
18:2 (Linoleic)	3.8 ± 0.5	6.7 ± 0.3	5.3 ± 0.8	7.0 ± 0.7	5.5 ± 0.4	11.7 ± 1.7	1.7 ± 0.2	13.4 ± 1.1
20:4 (Arachidonic)	7.6 ± 0.5	13.4 ± 0.4	15.9 ± 1.2	16.9 ± 0.9	3.8 ± 0.1	5.7 ± 0.8	4.7 ± 0.4	20.2 ± 1.4
22:4 (Docosatetraenoic)	0.2 ± 0.1	0.2 ± 0.05	0.3 ± 0.01	0.2 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.7 ± 0.1
22:5 (Docosapentaenoic)	0.4 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.01	1.1 ± 0.1
Linolenic type (ω3)								
18:3 (Linolenic)	0.1 ± 0.02	0.1 ± 0.02	0.0	< 0.1	2.0 ± 0.9	2.6 ± 1.2	0.1 ± 0.05	< 0.1
20:5 (Eicosapentaenoic)	0.5 ± 0.2	0.4 ± 0.05	0.2 ± 0.05	0.1 ± 0.02	7.1 ± 0.7	7.8 ± 1.7	7.4 ± 0.5	0.1 ± 0.02
22:5 (Docosapentaenoic)	1.1 ± 0.7	0.4 ± 0.1	0.5 ± 0.2	0.3 ± 0.05	1.7 ± 0.1	1.9 ± 0.4	1.8 ± 0.1	0.8 ± 0.3
22:6 (Docosahexaenoic)	3.5 ± 0.7	2.9 ± 0.5	2.2 ± 0.2	1.6 ± 0.1	5.1 ± 0.4	3.2 ± 0.6	6.9 ± 0.6	2.0 ± 0.2

[1] Rats were kept 12 to 17 weeks on diets. [2] Numbers of samples in parentheses. [3] Percent of lipid ± SE.

resulted in considerable substitution of ω3 fatty acids having 5 and 6 double bonds for ω6 EFA. The former totaled about 13 to 16% of the fatty acids, as compared with about 2 to 5% in the other experimental groups. Animals fed beef fat or low levels of corn oil showed varying amounts of ω9 eicosatrienoic acid, but the triene-to-tetraene ratio (31), indicative of EFA deficiency, was well under 0.4 in all animals except those fed 7% beef fat. Two groups of rats whose diets furnished about 0.55% of calories as linoleic acid (0.5% corn oil versus 0.3% corn oil + 6.7% beef fat) showed similar fatty acid patterns in liver phospholipids, suggesting that the level of the dietary lipid per se did not influence the fatty acid patterns when both diets furnished the same amount of linoleic acid. Feeding 2% linseed oil + 5% cod liver oil, however, resulted in a considerable depression of ω6 essential fatty acids and a predominance of ω3 or linolenic type fatty acids in the liver phospholipids, in comparison with the group fed 0.8% corn oil. Both diets furnished about 0.86% of their calories as linoleic acid, but in this case ω3 fatty acids substituted for much of the EFA in the tissue sites.

Similar differences were also observed in brain fatty acids among various experimental groups but to a much lesser degree than in liver phospholipids. Four ω6 EFAs constituted about 15% of the fatty acids of brain lipids from animals fed 7% corn oil, as compared with 11 to 12% in animals fed beef fat or low levels of corn oil, about 10% in rats fed 7% linseed oil, and only 8% in the two groups whose diets included cod liver oil. Omega-3 or linolenic type fatty acids made up from 12.3 to 15.9% of the fatty acids in brain lipids from animals fed linseed or cod liver oil, 9.2% in rats fed 7% corn oil, and 5.9 to 8.8% in animals fed beef fat or low levels of corn oil. Omega-9 eicosatrienoic acid appeared only in brains of rats fed 7% beef fat, 6.7% beef fat + 0.3% corn oil or 0.5% corn oil. These diets furnished the three lowest levels of PUFA.

No differences were seen in liver lipid (4.1 to 5.1%), in nitrogen levels in brain (1.79 to 1.95%) and liver (2.76 to 3.02%), in RNA in brain (2.51 to 2.65 mg/g) and liver (10.36 to 11.48 mg/g),

or in DNA in brain (1.99 to 2.38 mg/g) and liver (2.6 to 2.9 mg/g) among the experimental groups which could suggest that the effects of the dietary variables upon the enzymes might be due to tissue dilution.

In two experiments shown in table 3, 5-HTPD in liver was significantly lower in animals fed either beef fat or 0.5% corn oil. No differences were found in 5-HTPD activities in brain among the experimental groups. These averaged about 0.23 μmole/g/hour. The MAO and 5-HTPD activities were also evaluated in livers from rats fed diets which provided at least a minimum amount of EFA (table 3). Liver 5-HTPD was significantly lower in animals fed 1% corn oil, or 6.5% beef fat + 0.5% corn oil, in comparison with rats fed 7% corn oil. These two diets furnished about 1.2 and 0.95%, respectively, of their calories as linoleic acid. The lowest activity was observed in rats fed 7% beef fat, whose diet furnished about 0.28% of calories as linoleic acid. Liver MAO was significantly higher in rats fed 6.5% cod liver oil + 0.5% corn oil in comparison with the other experimental groups. This diet furnishes about 3.0% of calories as ω3 PUFA and about 0.8% of calories as linoleic acid, as well as about 0.6% as arachidonic acid, most of which appeared to be ω6 or essential.

TABLE 3

5-HTPD and MAO in livers from rats fed various lipids

Dietary lipid [1]	5-HTPD	MAO [2]
	μmoles/g/hr	
Exp. 1	(8) [3]	
7% Cod liver oil	4.76 ± 0.27 [4]	—
15% Corn oil	4.95 ± 0.17	—
15% Beef fat	3.27 ± 0.17 [5]	—
Exp. 2	(6)	
10% Corn oil	4.78 ± 0.22	—
2% Corn oil	4.42 ± 0.27	—
1% Corn oil	4.10 ± 0.29	—
0.5% Corn oil	3.44 ± 0.44 [5]	—
10% Beef fat	2.99 ± 0.20 [5]	—
Exp. 3	(10)	(7)
6.5% Cod liver oil — 0.5% corn oil	4.56 ± 0.16	19.3 ± 0.7
7% Corn oil	4.87 ± 0.21	16.7 ± 0.4 [8]
1% Corn oil	4.26 ± 0.14 [6]	15.8 ± 0.5 [9]
6.5% Beef fat — 0.5% corn oil	3.78 ± 0.15 [7]	15.6 ± 0.7 [9]
7% Beef fat	2.99 ± 0.16 [7]	15.4 ± 0.7 [9]

[1] In exp. 1, rats were kept for 13 to 15 weeks, and in exp. 2, for 4 to 10 weeks on diets isocaloric with group A from table 1. In exp. 3, rats were kept for 9 to 15 weeks on diets isocaloric with group B.
[2] Tryptamine-2-^{14}C as substrate.
[3] Numbers of animals per group are in parentheses.
[4] Mean ± SE.
[5] $P < 0.01$ that differences from groups fed 7% cod liver oil or 10% corn oil, respectively, are due to chance.
[6] $P < 0.05$ that differences from group fed 7% corn oil are due to chance.
[7] $P < 0.001$ that differences from group fed 7% corn oil are due to chance.
[8] $P < 0.02$ that differences from group fed 6.5% cod liver oil are due to chance.
[9] $P < 0.01$ that differences from group fed 6.5% cod liver oil are due to chance.

Incubation of liver homogenates from animals in experiment 2 with 1×10^{-5} M and 3×10^{-5} M concentrations of α-methyl-DOPA resulted in inhibitions of 17 and 33%, respectively, while 6×10^{-6} M and 2×10^{-5} M concentration of α-methyl-DOPA inhibited brain 5-HTPD by 30 and 46%. No differences were observed in percent inhibition by this drug among rats fed different diets.

The effects of various dietary lipids upon 5-HTPD, MAO, COMT, and 5-HTP metabolism are illustrated in table 4. Liver 5-HTPD and 5-HTP metabolism rates were significantly lower in rats fed beef fat or low levels of corn oil than in rats fed 7% cod liver oil or 7% corn oil. Highest activities of liver MAO, determined by two procedures, were observed in animals fed diets containing cod liver oil, whereas the lowest values were found in animals fed beef fat or low levels of corn oil. Liver COMT, however, was significantly lower in rats fed 7% cod liver oil, or 2% linseed oil + 5% cod liver oil, in comparison with the other groups not given cod liver oil. Metabolism of 5-HTP by liver homogenates by way of serotonin resulted primarily in the formation of 5-HIAA plus small amounts of neutral derivatives. Highest rates of 5-HTP utilization and 5-HIAA formation were obtained with liver samples from rats fed 7% levels of corn oil, linseed oil, or cod liver oil, which furnished high amounts of PUFA. These differences, to a lesser degree, were also seen in the formation of the neutral metabolites. Accumulation of serotonin was significantly less only with liver from rats given 7% beef fat. This is not surprising, since in other groups, the differences in decarboxylation to serotonin are probably cancelled by the corresponding oxidation of seroto-

TABLE 4

5-HTPD, MAO, COMT and 5-HTP metabolism in livers from rats fed various lipids. Relative effects of lipid levels and essential (ω6) and nonessential (ω3) fatty acids

Dietary lipid [1]	Calories as linoleic acid	5-HTPD	COMT	MAO		5-HTP metabolism in liver, 30 min			
				Tryptamine-2-^{14}C	Kynuramine	5-HTP	5-HT	5-HIAA	Neutrals
	%	*μmoles/g/hr*	*μmoles/g/hr*	*μmoles/g/hr*	*μmoles/g/hr*	*% decrease*	*% increase*	*% increase*	*% increase*
7% Cod liver oil	0.5	3.60 ± 0.22 [2]	26.5 ± 0.4 [6]	19.8 ± 0.7	34.8 ± 0.9	28.6 ± 0.3	6.6 ± 0.4	17.7 ± 1.0	1.89 ± 0.37
7% Corn oil	7.5	3.50 ± 0.19	31.0 ± 1.0	17.9 ± 0.9	34.7 ± 0.8	28.0 ± 1.3	6.6 ± 0.5	17.8 ± 0.9	1.51 ± 0.31
7% Linseed oil	1.8	3.07 ± 0.17	32.6 ± 0.8	19.1 ± 0.6	34.7 ± 0.4	28.1 ± 1.5	5.8 ± 0.3	17.2 ± 1.1	1.74 ± 0.40
2% Linseed oil + 5% cod liver oil	0.86	3.71 ± 0.14	28.6 ± 1.4 [7]	19.5 ± 0.8	35.3 ± 0.5	27.3 ± 1.2	6.1 ± 0.2	18.0 ± 1.1	2.04 ± 0.43
0.8% Corn oil	0.86	2.90 ± 0.21 [3]	33.0 ± 1.6	15.9 ± 0.7 [4]	28.8 ± 1.0 [5]	22.8 ± 1.3 [5]	5.9 ± 0.2	13.6 ± 1.1 [3]	0.78 ± 0.12 [3]
0.5% Corn oil	0.55	2.48 ± 0.16 [5]	33.7 ± 1.0	14.8 ± 0.8 [5]	27.8 ± 0.5 [5]	23.0 ± 1.4 [4]	6.0 ± 0.3	13.4 ± 0.8 [4]	1.23 ± 0.24
0.3% Corn oil + 6.7% beef fat	0.55	2.76 ± 0.15 [4]	33.2 ± 0.6	16.8 ± 0.7 [4]	30.8 ± 0.9 [4]	23.7 ± 1.7 [3]	6.1 ± 0.3	13.9 ± 1.2 [3]	1.04 ± 0.17 [3]
7% Beef fat	0.25	2.27 ± 0.11 [5]	33.7 ± 1.3	15.2 ± 0.6 [5]	27.1 ± 0.7 [5]	21.4 ± 1.7 [5]	5.4 ± 0.4 [4]	11.3 ± 0.7 [5]	0.90 ± 0.15 [4]

[1] Isocaloric with group B diets in table 1. Animals were kept from 13 to 23 weeks on diets.
[2] Mean ± SE.
[3] $P < 0.05$ that differeuces from group fed 7% cod liver oil are due to chance.
[4] $P < 0.01$ that differences from group fed 7% cod liver oil are due to chance.
[5] $P < 0.001$ that differences from group fed 7% cod liver oil are due to chance.
[6] $P < 0.001$ that differences from other groups not fed cod liver oil are due to chance.
[7] $P < 0.05$ that differences from four other groups fed beef fat or low levels of corn oil are due to chance.

nin by MAO. Liver MAO, 5-HTPD, and 5-HTP metabolism in rats fed 0.3% corn oil + 6.7% beef fat were slightly higher but not significantly higher than in animals fed 0.5% corn oil. Both diets furnished about 0.55% of their calories as linoleic acid. The same activities in livers from animals fed 2% linseed oil + 5% cod liver oil were, however, significantly higher ($P < 0.01$) than in rats fed 0.8% corn oil. Both groups of rats were given 0.86% of their calories as linoleic acid, but animals fed 2% linseed oil + 5% cod liver oil also received about 4.8% of their calories as ω3 PUFA, including about 2.4% as linolenic acid.

No differences were observed in 5-HTPD, MAO, or COMT activities in brains of rats from the different experimental groups. The activities in brain for 5-HTPD were, 0.17 to 0.22 μmole/g/hour; for MAO with tryptamine-2-^{14}C, 4.9 to 5.3 μmoles/g/hour; for MAO with kynuramine, 7.2 to 7.7 μmoles/g/hour; and for COMT, 0.49 to 0.51 μmole/g/hour.

Liver 5-HTPD was inhibited from 44 to 53% by 2×10^{-5} M NSD-1055 and 33 to 38% by 1×10^{-4} M NSD-1034, but no significant differences were observed in the effectiveness of these drugs among the experimental groups. Similarly, brain decarboxylase was inhibited 60 to 69% by 5×10^{-5} M NSD-1055 and 28 to 36% by 1×10^{-4} M NSD-1034, with no significant differences among the groups. No significant differences were found in the inhibition of MAO by JB-516 (Catron) or by iproniazid in liver and in brain among the experimental groups. Liver MAO was inhibited 23 to 30% by 1×10^{-6} M JB-516 and 55 to 62% by 5×10^{-4} M iproniazid. Brain MAO was inhibited 30 to 34% by 1×10^{-6} M JB-516 and 49 to 53% by 5×10^{-4} M iproniazid.

DISCUSSION

The data reported here support the hypothesis that fatty acid changes in the tissue structures can affect subcellular functions. It is possible that altering the fatty acid compositions of the lipoproteins on enzyme-supporting structures could affect some of the particle-bound enzymes, such as MAO, which has been reported to be primarily bound to mitochondria (32, 33). Similar changes might be observed concerning 5-HTPD, although there is only slight evidence that this enzyme might be loosely bound rather than soluble (33, 34), and these studies were done with brain rather than liver. In a preliminary investigation in our laboratory, over 90% of the 5-HTPD activity in liver was found in the supernatant fraction. COMT, however, has been described as a soluble enzyme (35), and the type of differences in MAO and 5-HTPD activities found among the experimental groups were not observed with COMT. The lower COMT activities in liver from animals whose diets included cod liver oil may be due to some factor other than the fatty acid content, since no inhibition of COMT was found in rats fed 7% linseed oil, whereas both linseed oil and cod liver oil in the diet led to increased tissue levels of ω3 PUFA at the expense of ω6 EFA. It should be noted, however, that a considerable increase in ω3 docosahexaenoic acid (22:6) in liver phospholipids was found only in animals fed cod liver oil.

The level of dietary fat per se did not appear to influence the activities of the enzymes discussed here. Feeding either 0.5% corn oil or 0.3% corn oil + 6.7% beef fat resulted in similar fatty acid profiles in liver phospholipids and in similar enzyme activities. Both diets furnished 0.55% of the calories as linoleic acid.

No differences were found in the activities of various drug inhibitors upon MAO and 5-HTPD among the experimental groups. The percentages of inhibition of the enzymes were the same, regardless of the uninhibited levels of activity found in the livers.

Brain activities of MAO and 5-HTPD did not differ among the experimental groups. Small differences were found in fatty acid compositions, but on the whole, brain lipids appeared to be much more resistant to changes in response to a difference in the dietary lipid.

Cellular and subcellular membranes have been described as consisting of various forms of layers of lipoproteins with phospholipid or hydrophobic structures in the leaflets (36, 37). Differences in activities reported here could be related either to changes in the enzyme properties resulting from altered fatty acid compositions

in the lipoproteins, or to changes in the rate of transport of substances through subcellular membranes. In any case, the data reported here serve to call attention to some of the nutritional factors which could easily affect biochemical and pharmacological variables at the cellular and subcellular level.

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Effects of Retinoic Acid and Progesterone on Reproductive Performance in Retinol-deficient Female Rats [1,2]

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ABSTRACT It was desired to determine whether administration of retinoic acid, or a combination of retinoic acid plus progesterone, could repair the defects in reproductive performance which occur in retinol-deficient female rats. A reliable degree of retinol deficiency was achieved in virgin female albino rats by feeding a retinol-replete diet from weaning until attainment of a body weight of 80 g, followed by a retinol-free diet until the rats reached 200 g and were mated. One group of 60 rats was fed an unsupplemented retinol-free diet, and two other groups of 48 and 60 rats, respectively, were additionally given all-*trans* retinoic acid by mixing it into the diet (10 μg/g) or by medicine dropper (375 μg/rat/week). Supplementation of the retinol-free diet with retinoic acid improved weight gains and restored regular estrous cycles in nonpregnant rats and also improved rates of mating and conception. Administration of retinoic acid by dropper was more effective than admixture in the diet. Neither retinoic acid alone nor in combination with 5 mg progesterone/rat/day from the day of conception prevented resorption or death of fetuses. Hemorrhage of the labyrinth and necrosis of the junctional zone of the placenta were characteristic antecedents of late fetal death. Although retinoic acid did not prevent resorption of fetuses in retinol-deficient dams, it supported formation of chorio-allantoic placentas and postponed the time of fetal resorption until approximately day 16 of gestation.

Considerable attention has been focused on the relationship between deficiency of vitamin A and reproductive failure in experimental animals (1–6). Mason's experiments in particular (3, 4) present a classic picture of reproductive failure in rats as characterized by fetal resorption, stillbirths, congenital malformations, prolonged labor, irregular estrous cycles, and cornification of the vagina which tends to obscure estrous cycles. It has been suggested that failure of reproduction might be a nonspecific resultant of general debility of the retinol-deficient animal (7) since deficiency of retinol is known to cause a variety of pathological disorders such as stunted or abnormal growth, decreased resistance to infection, lesions of the nervous system (8), keratinization of epithelial cells (9), and suppression of steroid hormone biosynthesis (10). This suggestion has been disputed on the ground that reproductive failures have resulted even when the animals have been fed retinoic acid, a substance known to correct the above abnormalities while not supporting vision and reproduction (11–20).

The earliest abnormality observed during pregnancy in retinol-deficient rats receiving retinoic acid is necrosis of the junctional zone of the placenta which later spreads to the labyrinth (14). That placental damage is associated with poor reproductive performance in retinol-deficient rats suggests that such rats may be suffering from insufficiency or imbalance of steroid hormones. This suggestion is supported by the fact that the fetal resorption occurring in retinol-deficient rats also occurs in reproductive failure caused by deficiency of ovarian progesterone (21–24) and that steroid hormone synthesis, especially conversion of pregnenolone to progesterone, is depressed in rats deficient in retinol (10).

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In the present work reproductive performance of retinol-deficient rats was examined with respect to rates of conception, formation of chorio-allantoic placentas, continued placental integrity, and delivery of live young. Retinoic acid was given to determine its effects upon the variables mentioned and to produce retinol-deficient rats not suffering from general growth suppression and debility. Progesterone was injected into retinol-deficient rats fed retinoic acid to determine whether this hormone could correct any remaining reproductive defects and permit the rats to carry gestation to a successful conclusion.

MATERIALS AND METHODS

A preliminary experiment was carried out with virgin, female albino rats of the Navy strain (raised in the Division of Nutrition) with the object of establishing standard conditions for the development of retinol deficiency and methods for the introduction of retinoic acid. A second experiment was conducted, under the established conditions, in which the effects of progesterone were studied in rats of the same strain fed a retinol-free diet supplemented with retinoic acid. All experiments were carried out in air conditioned animal quarters kept at 21 to 27°, with the females caged in pairs.

Preliminary experiment. One hundred and seven weanling females with initial weights ranging from 30 to 40 g were used. Daily records were kept of body weight, and vaginal smears were recorded daily starting 2 weeks before the desired date of mating. The rats were fed a commercial laboratory ration [3] until they reached a body weight of approximately 80 g. Thereafter they were fed a retinol-free diet, vitamin A test diet, USP.[4] Group 1 was continued with this retinol-free diet; group 2 was fed the retinol-free diet supplemented with crystalline all-*trans* retinoic acid,[5] 10 μg/g of diet (see table 1). No special care was taken to mix this diet at frequent intervals; unused retinoic acid was wrapped in light-proof foil and kept in an evacuated desiccator. Females of both groups were mated as soon as possible after they had been fed the retinol-free diet (with or without retinoic acid) for 75 days. The day on which sperm were found in the vaginal smear was designated as day zero of gestation.

Rats were killed with ether on day 10, 11, or 12 of gestation and at least six whole conceptuses from each animal were removed and examined grossly. Care was

[3] Purina Laboratory Chow Pellets, Ralston Purina Company, St. Louis.

[4] Diet composition: (in percent) USP salt mixture no. 2, 4; irradiated yeast, 8; starch, 65; vegetable oil, 5; vitamin-free casein, 18; viosterol, 0.0011.

[5] Eastman Organic Chemicals, Rochester, New York.

TABLE 1

Group designations of vitamin A-deficient rats in relation to diet and injection

Group	No. of rats	Diet	Daily injections
		Preliminary experiment	
1	59	Retinol-free diet	none
2	48	Retinol-free diet supplemented with all-*trans* retinoic acid; supplements were added by mixing 10 μg of retinoic acid/g of diet	none
		Progesterone experiment	
2-O	30	Same as group 1 diet but all-*trans* retinoic acid was dissolved in corn oil and administered through a medicine dropper 3 times weekly, each dose containing 125 μg, totaling 375 μg/rat/week	vehicle (corn oil) alone; intramuscular
2-P	30	Same as group 2-O	5 mg progesterone diluted in corn oil; intramuscular

taken to select only conceptuses that did not appear to be undergoing resorption. These were fixed in Bouin's solution for 3 to 4 days, sectioned at 7 μ, and stained with hematoxylin and eosin. At least 2 conceptuses from each dam were examined microscopically.

Progesterone experiment. Sixty females were fed the commercial laboratory ration until they reached a weight of approximately 80 g, and were then fed the retinol-free diet. All of these rats were given 375 μg of all-*trans* retinoic acid by medicine dropper in three weekly doses of 125 μg each. The retinoic acid was dissolved in a small amount of corn oil. This mode of administraton of retinoic acid was introduced to attain higher growth rates than could be obtained when retinoic acid was added to the diet. The rats were mated after 75 days of receiving this diet. Starting with day zero of gestation, 30 rats were injected intramuscularly with 0.2 ml corn oil (group 2-O) daily, and the remaining 30 rats (group 2-P) were injected intramuscularly with 5 mg progesterone in 0.2 ml of oil [6] daily. See table 1 for identification of groups of rats. Six rats from each of these groups were permitted to proceed to term and the others were killed on day 12, 14, 16, or 18 of gestation. Injections of vehicle or progesterone were stopped on day 20 of gestation for those rats that continued to term. Liver and blood serum were saved for analysis of vitamin A. Conceptuses were collected and examined as previously described. Placentas, rather than whole conceptuses, were collected from rats pregnant more than 12 days. Lipids were extracted from the liver (25), and both extracts and serum were analyzed for their content of retinol by a trifluoroacetic acid method (26). Serum and liver vitamin A concentrations were measured for rats fed laboratory chow and for rats fed the retinol-free diet with admixed retinoic acid for 75 days and rats fed the retinol-free diet and retinoic acid by medicine dropper for 75 to 100 days. Serum was prepared immediately after bleeding, and both serum samples and livers were stored frozen until ready for analysis 2 to 4 weeks later.

RESULTS

Weight gains and general appearance. Body weight gains are shown in figure 1. Rats fed the vitamin A-free diet (group 1) manifested most of the classic signs of deficiency of this vitamin. There was poor weight gain, followed by decline in body weight during the tenth week of feeding the retinol-free diet. Weight gains of group 2-O and 2-P rats were similar and were plotted as a single curve. This curve closely followed that for normal rats (those fed stock laboratory ration). However, the curve for rats of group 2, given retinoic acid in the diet, fell below the curves for normal rats and those given retinoic acid by dropper, more closely approached the curve for group 1. The weight gain curves for normal rats and those of groups 2-O

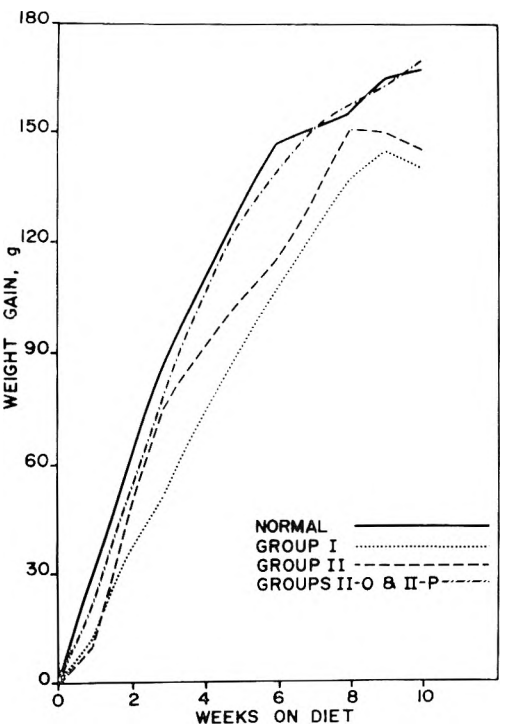

Fig. 1 Weight gains of female rats fed laboratory chow (normal), retinol-free diet (group 1), and retinol-free diet supplemented with retinoic acid, either mixed in the diet (group 2) or given by medicine dropper (groups 2-O and 2-P).

[6] Proluton, Schering Corporation, Bloomfield, New Jersey.

and 2-P continued to rise, whereas those for rats of groups 1 and 2 began to fall after the ninth week. The difference in weight gains of group 2 rats from those of groups 2-O and 2-P is ascribed to the difference in the mode of administration of the retinoic acid supplements. Administration by medicine dropper was more effective than admixture in the diet, possibly because retinoic acid in the diet was damaged by light. Skeletal deformities developed in some of group 1 rats. Such rats developed a hunchback appearance and peculiar gait as observed in dogs by Mellanby (27). Some of these rats had coughing spells, and others had convulsive seizures lasting up to 1 minute. These signs of vitamin A deficiency were completely absent in normal rats and those of groups 2, 2-O, and 2-P. No ocular changes were observed, possibly because the length of time during which the rats were fed the retinol-free diet was not sufficient for total depletion of vitamin A stores in the eye (28).

Estrous cycles and rates of conception. Estrous cycles of group 1 rats became irregular soon after the menarche. Later there was constant vaginal cornification, unrelated to any particular stage of the reproductive cycle, as evidenced by continuous presence of large numbers of cornified cells in the vaginal smears. Rates of conception, pseudopregnancy and failure to mate are shown in table 2. Rats were judged to have been pseudopregnant if they showed continued anestrus, gained weight to the same extent as pregnant animals, and displayed hyperemic ovaries and uteri, but no fetuses, at autopsy. Rats of group 1 showed the lowest rates of conception and the highest rates of pseudopregnancy and failure to mate. Administration of retinoic acid by dropper was slightly more effective than admixture in the diet in improving the rate of conception. Pseudopregnancies and failures to mate were rarer in rats given supplements of retinoic acid.

Microscopic examination. In examining conceptuses and placentas collected from rats fed the retinol-free diet, with or without retinoic acid, particular attention was paid to whether or not a true chorio-allantoic placenta had formed; and if so, whether any pathological processes could be observed; and if so, at what time they appeared. The criterion adopted for the presence of a chorio-allantoic placenta was the formation of fetal blood channels within the labyrinth as identified by the presence within them of nucleated fetal erythrocytes. The number of rats that showed true chorio-allantoic placental formation are set forth in table 3. Dietary, and especially oral administration of retinoic acid had a beneficial effect upon placental formation. Additional treatment with 5 mg progesterone/day gave no significant improvement beyond the result achieved with oral retinoic acid alone.

Placental lesions were seen in all groups of retinol-deficient rats. Hemorrhages of the junctional zone and within the labyrinth were commonly seen. At a later stage there was generalized necrosis of the junctional zone which led to gradual degeneration of the embryo. These findings are summarized in table 4.

Administration of retinoic acid in the diet tended to delay the onset of embryonic degeneration until about day 11 of gestation, whereas oral administration of this substance delayed the onset of most pathological changes until day 16. After this time, however, few if any conception sites

TABLE 2

Rates of conception, pseudopregnancy, and inability to mate in vitamin A-deficient rats

Group	Total no. of rats	Rats that conceived		Rats that became pseudopregnant		Rats that failed to mate	
		No.		No.		No.	
			%		%		%
1	59	18	30.5	32	54.3	9	15.2
2	48	34	70.8	14	29.2	0	
2-O	30	23	76.6	5	16.7	2	6.7
2-P	30	23	76.6	3	10.0	4	13.4

TABLE 3

Effect of dietary supplements of retinoic acid upon the development of chorio-allantoic placentas in vitamin A-deficient rats

Group	Retinoic acid fed	No. of days fed diet	No. of rats mated	No. of placental sites examined [1]	No. of sites where a chorio-allantoic placenta was:	
					Present [2]	Absent [2]
1	None	75–100	8	16	2	14
		100–125	3	6	—	6
		125–150	—	—	—	—
		150–175	1	2	1 [3]	1
2	10 μg/g diet	75–100	15	30	8	22
		100–125	6	12	2	10
		125–150	5	10	2	8
		150–175	2	4	—	—
2-O	375 μg/week by dropper	75–100	23	46	42	4
2-P	375 μg/week by dropper	75–100	23	46	44	2

[1] Sites were examined at autopsy on days 10 to 12 of gestation.
[2] See text for criterion of presence or absence of a chorio-allantoic placenta.
[3] Degenerating.

TABLE 4

Summary of pathological changes in vitamin A-deficient pregnant rats

Group	Day of gestation	No. of rats	No. of conceptuses examined	No. of conceptuses showing hemorrhagic junctional zone and labyrinth	No. of conceptuses showing necrosis of junctional zone	No. of conceptuses showing incipient embryonic degeneration
1	10	5	10	6	10	8
	11	5	10	9	10	3
	12	4	8	8	8	5
2	10	6	12	8	4	0
	11	9	18	14	18	4
	12	11	22	22	22	20
2-O	12	3	6	3	3	2
	14	6	12	4	4	2
	16	5	10	10	10	10
	18	6	12	12	12	12
2-P	12	1	2	1	1	0
	14	6	12	2	6	0
	16	6	12	8	8	8
	18	6	12	10	10	12

had escaped severe damage and embryos were destroyed. Administration of progesterone afforded extra protection to the embryo up to day 14 of gestation but not thereafter.

By day 16 of gestation, placental damage was extensive in rats of groups 2-O and 2-P, and nearly all embryos were degenerating. Placental damage at this time consisted mainly in loss of fetal blood channels and invasion of the former channels with maternal blood, which gave a hemorrhage-like appearance to the labyrinth. Figure 2 shows an 18-day placental labyrinth from a normal rat with intact fetal blood channels, and figure 3 shows the hemorrhagic appearance of an 18-day labyrinth from a rat of group 2-O fed the retinol-free diet supplemented with retinoic acid by medicine dropper.

In animals in which placental damage was extensive after day 16 of gestation, the fetuses were adversely affected among all groups of rats. Fetal degeneration was

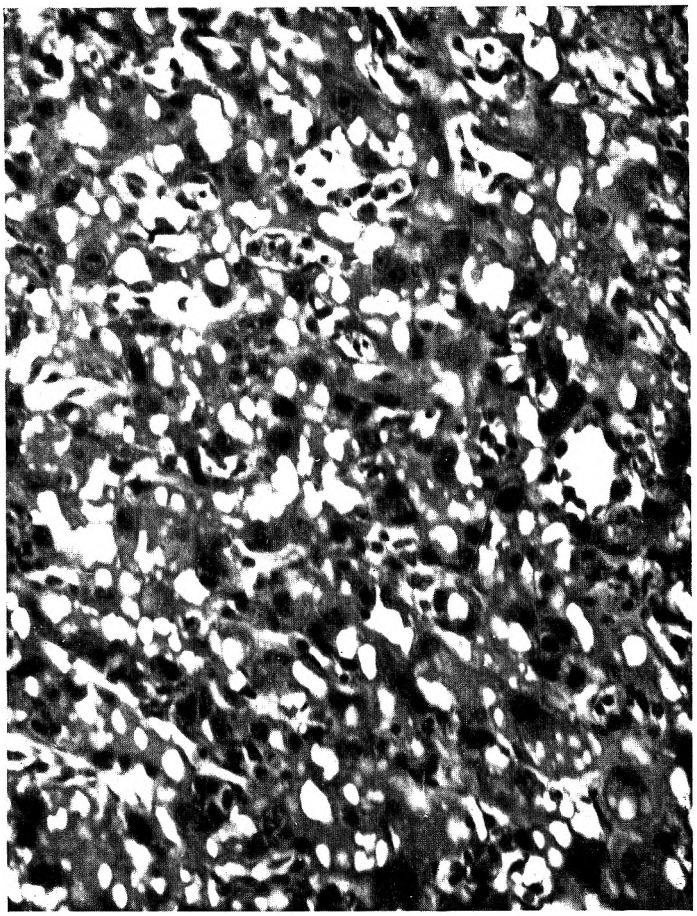

Fig. 2 Labyrinth of 18-day placenta from a normal rat. × 235.

recognized by the fuzzy appearance of neural tubes and somites. Toward the end of gestation in all retinol-deficient rats given retinoic acid (groups 2, 2-O, and 2-P) the placental labyrinths became either empty or filled with blood. It appeared that maternal-fetal exchange of nutrients had stopped. No fetuses in any retinol-deficient group survived to full term, though one dam of group 2-P (progesterone-injected) delivered two dead, malformed pups.

Serum and liver concentrations of vitamin A. Concentrations of retinol in the serum and liver of rats fed a replete diet and of pregnant rats fed retinol-free diets are shown in table 5. After a 75- to 100-day period of depletion, the serum concentrations of retinol fell to values only one-third as high as those of replete rats. Liver stores in the pregnant, retinol-deficient rats were too low to be detected by the method used.

DISCUSSION

Newton (29) showed in 1938 that pregnant rats fed a vitamin A-free diet after they had attained a weight of 35 to 100 g with a replete diet failed to complete gestation because of fetal resorption. If the deficiency diet was fed from weaning, failure to mate was the most common defect. In the present work, weanling rats were brought to 80-g body weight with laboratory ration and then fed a retinol-free diet, with or without supplements of retinoic acid. This procedure produced a reliable degree of retinol deficiency. Serum levels of vitamin A were consistently about one-third of normal, whereas liver stores were essentially depleted.

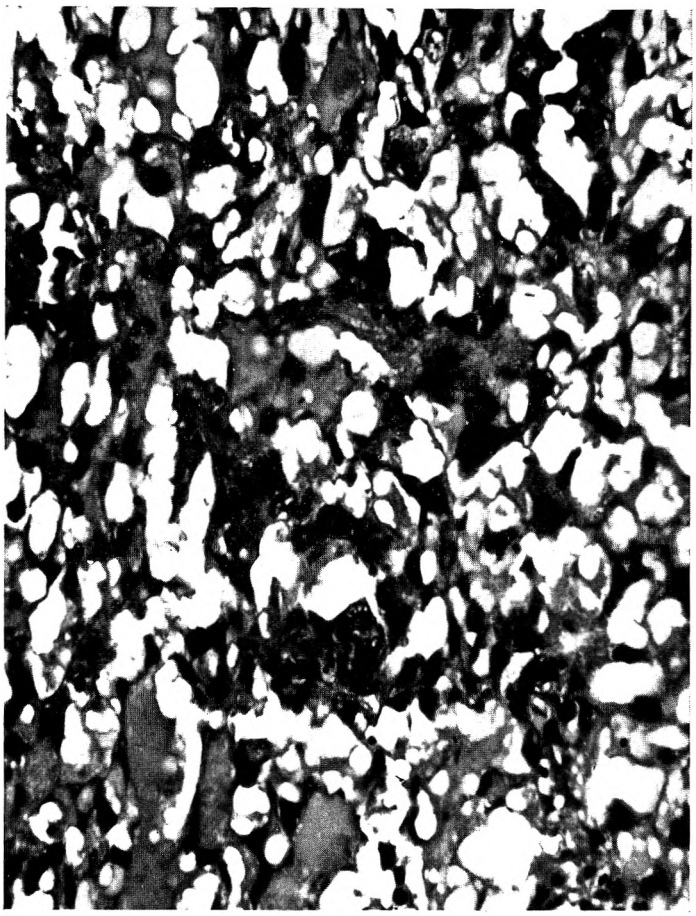

Fig. 3 Labyrinth of 18-day placenta from a retinol-deficient rat given retinoic acid by medicine dropper and injected with corn oil vehicle. While areas of maternal blood remain, no fetal red blood cells are seen. × 235.

TABLE 5

Serum concentrations and liver stores of vitamin A

Group	Diet	Injections	No. of days fed deficient diet	No. of rats	Vitamin A concn	
					Serum	Liver
					μg/100 ml	*μg/g*
Normal	Stock ration	none	—	6	33	3.0
1	Retinol-free	none	75	6	12	nd [1]
2-O	Retinol-free + oral retinoic acid	corn oil alone	75–100	30	10	nd
2-P	Retinol-free + oral retinoic acid	5 mg progesterone in corn oil; intramuscular	75–100	30	12	nd

[1] Not detected.

The present data demonstrate that retinoic acid in the absence of retinol provides nutritional support for improving weight gain, frequency of mating, and conception in the rat. A most striking effect of retinoic acid, in our hands, was its support of invasion of allantoic blood vessels into the labyrinth and thus formation of chorio-allantoic placentas. This effect was not further augmented by injection of 5 mg progesterone/day from conception.

Regardless of improvements in mating, conception, and formation of placentas, there was no success in obtaining live young from retinol-deficient dams even when both retinoic acid and progesterone were given. Failure to carry gestation to a successful conclusion was probably not ascribable to insufficient supply of retinoic acid, as the rats showed improved growth, mating and conception. These results are in accord with those of Juneja et al. (13), and of Thompson et al. (14, 30), who used either 10 μg of methyl retinoate/g diet or 700 μg/week of sodium retinoate in 2 doses of 350 μg mixed into the diet. In the present work one group of rats received 10 μg of crystalline all-*trans* retinoic acid/g diet (group 2). The overall food consumption of these rats averaged 5 g/day. Thus the weekly consumption of retinoic acid was 50 μg/day for 7 days, or 350 μg/week. This computation was used in selecting the dose of 375 μg/week by medicine dropper. This was only about half the dose of methyl retinoate used by Thompson and co-workers. Nevertheless, in neither study did retinoic acid support full-term gestation in the presence of a severe deficiency of retinol.

Other dietary manipulations are known to cause failure of gestation. The best-known example is that of vitamin E deficiency (31), but such failures also occur when the diet is lacking in pantothenic acid (32), pyridoxine (33), protein (34), or when there are multiple deficiencies of vitamins, minerals, and protein (35). It is notable that in all of these instances live young could be obtained if the dams had been given daily injections of progesterone in amounts up to 5 mg/day. Although injections of progesterone have been shown to be beneficial in vitamin A-deficient rabbits (36), neither progesterone alone as in the present study, or progesterone plus estrogen as used by Coward et al. (37), have led to birth of live young in retinol-deficient rats given retinoic acid. It is usually considered that a combination of 3 or 4 mg progesterone/day with 1 μg estrone/day will suffice to maintain pregnancy in rats ovariectomized in early pregnancy (23). In view of this maintenance requirement it is of interest that pregnancy apparently cannot be maintained in the retinol-deficient rat by similar doses of progesterone and estrogen, while these steroids have proved to be effective in rats deficient in other nutrients as listed above. The reason for these latter successes is not clear, especially in the absence of corroborating evidence that deficiency of pantothenic acid, pyridoxine, or protein leads to disturbed synthesis or removal of steroid hormones.

Although lack of retinol blocks the synthesis of progesterone from pregnenolone, this metabolic defect can be repaired under some conditions by retinoic acid (10). This finding emphasizes that fetal resorption or other features of reproductive failure in retinol-deficient rats are not necessarily caused by lack of specific steroid hormones or any other disturbances in hormone metabolism. The evidence reported here supports the concept of Thompson et al. (30) that there are three dissociable modes of action of vitamin A, namely, systemic, visual, and reproductive. It is evident that neither retinoic acid alone nor in combination with progesterone, in the doses used here can replace retinol in its specific action on reproduction. This action appears to be one of maintenance of the integrity of the conceptus from the time of formation of the chorio-allantoic placenta to term.

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Genesis of Esophageal Parakeratosis and Histologic Changes in the Testes of the Zinc-deficient Rat and Their Reversal by Zinc Repletion [1]

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ABSTRACT A histologic study of the early changes in the esophagus and testes was made in the zinc-deficient rat. Cellular changes in these tissues were described in 2 experiments comprising a total of 72 animals, extending to 44 days in the first study and 45 days in the second. In the first study, rats were fed a zinc-deficient diet (0.5 ppm) and compared with pair-fed and ad libitum controls fed the same diet supplemented with 30 ppm of zinc. The earliest changes in the esophagus of hyperkeratosis and parakeratosis were observed on day 16 of the experiment. In the testes of the zinc-deficient animals fed their diet for 23 days, there was a decreased number of spermatids with many seminiferous tubules appearing immature in comparison with those of the pair-fed controls. In zinc deficiency of the rat the transformation of spermatids to spermatozoa is inhibited, which appears to be the earliest histologic change in spermiogenesis. The animals in the second study were offered a low zinc diet (2.5 ppm) for 10 days, then fed a zinc-deficient diet (0.5 ppm) for 13 days, and were compared with pair-fed controls. Histology of the esophagi of 4 rats fed the low zinc diet for 10 days was similar to that of the pair-fed controls. Eleven deficient animals were repleted with 1 mg of zinc ($ZnSO_4 \cdot 7H_2O$) by intraperitoneal injection in the 2 experiments. In the first study, 3 rats were repleted for 3, 12, and 18 hours, respectively, for histology of the esophagi, and 2 rats were repleted for 14 days for histology of the testes. In the second, 6 rats were repleted for 24 hours and showed complete keratinization of their esophagi, compared with the progressive recovery of the lesion in the 3 rats of the first study. No recovery was observed in the testes. Zinc determinations from pooled serum of 15 rats in the first study representing the deficient, repleted, pair-fed and ad libitum groups had values from 82.5 to 97.5 μg/100 ml in the repleted and deficient, and 123 to 192 μg/100 ml in the controls.

Rats fed a zinc-deficient diet for 7 to 8 weeks show parakeratosis of the esophagus and nearly complete atrophy of the testes (1–3). Genesis of these lesions has not been studied, and trials to reverse these changes by repletion are relatively few (2, 3). The present report describes the early histologic changes in the esophagus and testes of zinc-deficient rats as studied by light and phase microscopy, and the effect of zinc repletion.

METHODS

In the first experiment, 46 male, 21-day old rats of the Sprague-Dawley strain that averaged 44.8 g in weight were divided into 3 groups. These groups were: 20 animals fed a zinc-deficient diet (0.5 ppm zinc) containing in grams per 100 g: zinc-low casein,[2] 20 (4); cottonseed oil,[3] 10; vitamin-sucrose mix,[4] 5; mineral mix,[5] 3.55; choline chloride, 0.15; and sucrose,[6] 61.3; a second pair-fed group of 20 animals was offered the same diet supplemented with $ZnSO_4 \cdot 7H_2O$ to provide an additional 30 ppm of zinc; and, a third group of 6 animals was fed the zinc-supplemented diet ad libitum. All animals were housed in plastic cages designed and

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[2] Casein purified; Nutritional Biochemicals Corporation, Cleveland.

[3] Vitamin A, 1000 IU (Aquasol A, U. S. Vitamin and Pharmaceutical Corporation, New York), vitamin D_2, 125 IU (Drisdol, Winthrop Laboratories, New York), and α-tocopherol, 60 mg (Nutritional Biochemicals Corporation) were added to 10 g cottonseed oil (Wesson Oil, Hunt-Wesson Foods, Fullerton, California).

[4] Vitamin-sucrose mix, mg/kg of mix: thiamine·HCl, 200; riboflavin, 120; pyridoxine·HCl, 80; Ca pantothenate, 320; biotin, 4; nicotinic acid, 300; folic acid, 10; vitamin B_{12}, 0.4; menadione, 6.6; and sucrose to make 1000 g.

made locally.[7] Diet was fed in plastic cups and deionized water was provided ad libitum from plastic bottles. Starting on the sixth day, the daily food intake of the pair-fed group was adjusted to equal the mean intakes of the animals fed the zinc-deficient diet.

The esophagi from 20 zinc-deficient animals and 20 pair-fed controls killed at various times between days 7 and 44 of the experiment were examined by light microscopy (table 1). Three zinc-deficient rats were repleted on days 29 and 30 of the experiment by intraperitoneal injection of 1 mg of zinc ($ZnSO_4 \cdot 7H_2O$) 3, 12, and 18 hours before killing, and their esophagi were examined by light and phase microscopy. One testis from each of 6 zinc-deficient animals and 6 pair-fed controls killed on days 9, 16, and 23 of the experiment was examined histologically, and the other testis was removed for zinc analysis. On day 30, 2 zinc-deficient rats were repleted with 1 mg of zinc ($ZnSO_4 \cdot 7H_2O$) by intraperitoneal injection after hemicastration. They were fed the control diet for 14 days before removal of the other testis.

TABLE 1

Comparison of body weights and incidence of esophageal parakeratosis in rats fed a zinc-deficient, pair-fed and ad libitum control diet

No. of rats	Days fed diet	Mean body wt when killed	Parakeratosis of esophagus [1]
		g	
	Zinc-deficient		
3	7	54.1	0
2	9	66.0	0
2	16	65.1	1
3	17	67.4	2
3	22	72.6	3
2	23	68.7	2
2 [2]	29	80.1	2
1 [2]	30	67.9	1
2 [3]	44	141.5	0
	Pair-fed control		
3	7	55.4	0
2	9	66.1	0
2	16	82.8	0
3	17	85.1	0
3	22	90.6	0
2	23	87.0	0
2	29	94.7	0
1	30	89.2	0
2	44	150.3	0
	Ad libitum control		
1	7	53.5	—
2	17	85.7	—
2	20	110.5	—
1	30	196.4	—

[1] Parakeratosis was based on the persistence of nuclei in the stratum corneum.

[2] Three rats repleted by intraperitoneal injection of 1 mg of zinc ($ZnSO_4 \cdot 7H_2O$) and killed 3, 12 and 18 hours thereafter.

[3] Two rats repleted with zinc as above after hemicastration on day 30 and fed the control diet ad libitum for 14 days.

The esophagi for light and phase microscopy were either fixed in 10% neutral formalin, embedded in Paraplast [8] and cut into 6-μ sections, or in 2% osmium tetroxide,[9] previously diluted with 0.3 M phosphate buffer to a final molarity of 350 mmoles, embedded in epoxy resin,[10] and cut into 1.4-μ sections. Testes were fixed in Bouin's fluid, embedded in Paraplast and cut into 7-μ sections.

Zinc was determined in serum and testes by atomic absorption spectroscopy. Testes were prepared for analyses by a wet-ashing procedure.[11] Blood was withdrawn from the aorta of 3 to 5 rats in each group, and equal amounts of serum from each rat were pooled. The pooled sera was diluted 1:1 with deionized water for analysis.

In the second experiment, a zinc-low diet (2.5 ppm) was fed to 26 male, 21-day-old Sprague-Dawley rats for 10 days. Four of these rats were killed on the tenth day of the experiment, and their esophagi examined by light microscopy. There was no evidence of parakeratosis at this time. The remaining rats were divided into 2 groups: 14 rats were fed the zinc-deficient diet (0.5 ppm), and 8 were pair-fed the same diet supplemented with 30 ppm of zinc ($ZnSO_4 \cdot 7H_2O$). Six of the deficient animals were repleted on the thirteenth day of the study by intraperitoneal injection with 1 mg of zinc ($ZnSO_4 \cdot 7H_2O$) 24 hours before killing, and their esophagi were examined histologically and compared with those of 6 pair-fed controls.

[5] Each 3.55 g of mineral mix contained: (g) $CaHPO_4$, 2.58; KCl, 0.343; Na_2CO_3, 0.115; $MgSO_4 \cdot 7H_2O$, 0.405; $FeSO_4 \cdot 7H_2O$, 0.06; $MnSO_4 \cdot H_2O$, 0.031; $CoCl_2 \cdot 6H_2O$, 0.004; $CuSO_4 \cdot 6H_2O$, 0.006; KI, 0.0004; and NaF, 0.0008.

[6] Commercial cane sugar. Godchaux Sugar Refining Company, New Orleans, Louisiana.

[7] Economy Plastics, Nashville, Tennessee.

[8] Fisher Scientific Company, New York.

[9] See footnote 8.

[10] Araldite, Ciba Products Company, Summit, New Jersey.

[11] Analytical Methods for Atomic Absorption Spectrophotometer Manual 990-9461, 1964, Perkin-Elmer Corporation, Norwalk, Connecticut.

RESULTS

Growth curves of the zinc-deficient and control rats are shown in figure 1. Parakeratosis of the esophageal mucosa was noted in 11 of 18 zinc-deficient rats. It was first evident on day 16 and thereafter, with the exception of 2 animals killed on days 16 and 17 (table 1). The esophagi in the latter 2 rats were hyperkeratotic. In the pair-fed controls, the esophageal mucosa averaged 4 to 5 layers in thickness, and the keratohyalin granules were in stellate-shaped cells subjacent to the stratum corneum (fig. 3 (1)). Excluding the parakeratotic zone, the esophagi of the zinc-deficient animals that showed imperfect keratinization on days 16 and 17 were 5 to 6 cell layers in mucosal thickness and the morphology of the cells in the granular layer were mostly polyhedral in shape with ovoid nuclei. The zinc-deficient rats killed on days 22 and 23 showed a mucosal thickness of 11 to 12 layers under the parakeratotic zone, and the keratohyalin granules were in polyhedral cells located in the mid-portion of these layers. These early changes of esophageal parakeratosis are similar to those described by Follis et al. (1) in rats fed a zinc-deficient diet for 34 to 74 days. Follis stated, however, that the stratum overlying the basal cells was only 6 to 8 cell layers thick. It is probable that the diet used by these workers contained more zinc than the one used in the present study.

In the esophageal mucosa of the rat repleted for 3 hours, there was a dense zone of 5 to 6 layers of stellate-shaped cells above the stratum basal with a less dense zone of 8 to 9 layers of polyhedral cells overlying it (fig. 3 (2)). Keratohyalin granules were sparse at the junction of these 2 zones. In the rat repleted for 12 hours, the esophageal changes were comparable, but the mucosa was 9 to 10 cell layers in thickness below the parakeratotic zone with keratohyalin granules frequent in numbers in the mid-portion of these layers. Unexpectedly, in the esophageal mucosa of the rat repleted for 18 hours, there appeared a discrete layer between the parakeratotic zone and the outer-most cell layer (fig. 3 (3)). There was an increase in number of keratohyalin granules compared with the pair-fed control. Of equal importance was the presence of stellate-shaped cells with keratohyalin granules approximate to the stratum corneum. The esophageal mucosa was 7 to 8 cell layers in thickness below the parakeratotic zone. In the second experiment, the esophageal mucosa of the 6 zinc-repleted rats was comparable to the 6 pair-fed controls after 24 hours of repletion, except for the parakeratotic zone in the lumen and the presence of more keratohyalin granules (fig. 3 (4)).

When rats were fed the zinc-deficient diet or the pair-fed control diet for 1 to 2

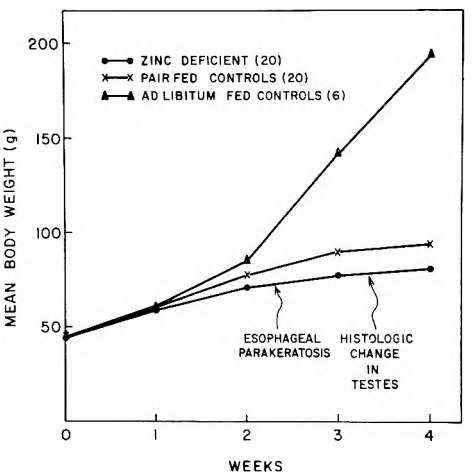

Fig. 1 Growth curves of zinc-deficient and control rats.

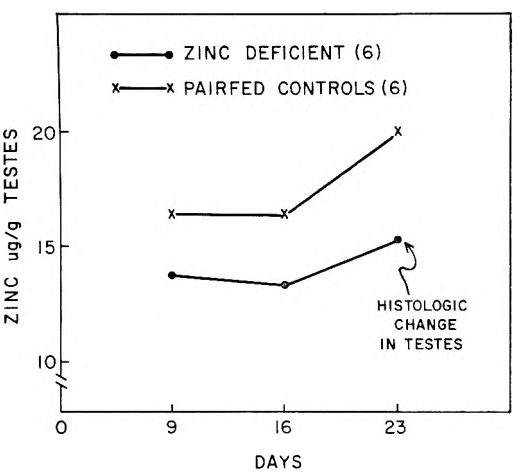

Fig. 2 Mean zinc concentration in testes of 2 zinc-deficient and 2 pair-fed controls on days 9, 16, and 23.

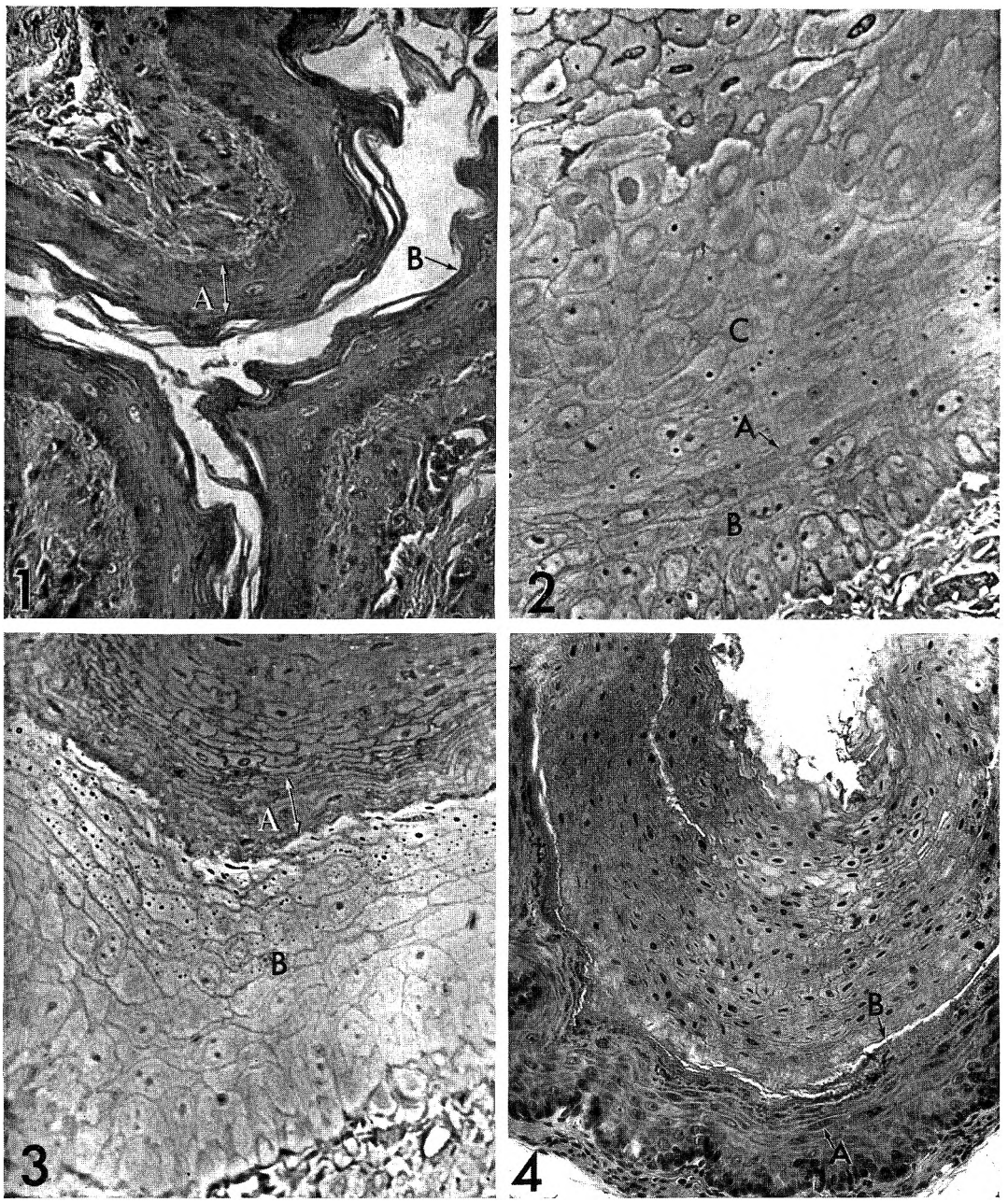

Fig. 3 (1) Pair-fed control esophagus of a rat showing a mucosal thickness of 4 to 5 cell layers (A) with the normal appearance of a stratum corneum (B). Light microscopy. (2) Esophagus from a zinc-deficient rat repleted with 1 mg of zinc 3 hours before killing. Note junction (A) between the 2 zones and sparse number of keratohyalin granules. Cells are more stellate-shaped in lower, dense zone (B) compared to the polyhedral cells (C) in the upper layers. Unstained. Phase microscopy. (3) Esophagus of a zinc-deficient rat repleted with 1 mg of zinc 18 hours before killing, with a discrete layer between the stratum granulosum and parakeratotic zone (A). Keratohyalin granules are frequent in number 3 to 4 cell layers below the keratinized layer (B). Unstained. Phase microscopy. (4) Esophagus from a zinc-deficient rat repleted with 1 mg of zinc 24 hours before killing. Mucosa is 6 to 7 cell layers in thickness. Stellate-shaped nuclei are oriented parallel to stratum corneum (A) with keratohyalin granules subjacent to this layer. Note separation of stratum corneum and parakeratotic zone (B). Light microscopy.

weeks, there was no difference in the histology of the testes. Multi-nucleated cells and spermatocytes shed from the germinal epithelium were seen in the lumen of the seminiferous tubules and the epididymal canal, which is a normal feature in rats of this age (5). On day 23 of the experiment the overall picture of the testes in both groups was similar. A few degenerate tubules were seen, but they were in the same proportion in each group. However, in the zinc-deficient animals the number of elongated spermatids was reduced in most tubules in stage 3 of Roosen-Runge's classification of the seminiferous epithelium cycle (6). This was confirmed by the absence of sperm in the lumen of the epididymal canal of the zinc-deficient rats in contrast to the many sperm in the epididymides of the pair-fed controls (fig. 4 (5)). On day 30, the reduction in the number of elongated spermatids was even more striking (fig. 4 (7)). There were a number of tubules which showed a non-specific type of degeneration with pyknotic spheres, exfoliation of cells, and the formation of multi-nucleated spermatids (fig. 5 (9)). In the epididymis of the zinc-deficient rat, very few sperm could be seen compared with those of the pair-fed controls which were packed with sperm. After repletion for 2 weeks, there was no amelioration and more tubules appeared to be severely damaged, shrunken, and lined by spermatogonia and spermatocytes (fig. 5 (12)). However, tubules subjacent to the tunica albuginea tended to be less affected than those deep in the testes, and even there, normal tubules were close to atrophic ones (fig. 5 (11)).

Five rats fed the deficient diet for 22 or 23 days had serum zinc concentrations of 97.5 μg/100 ml, and 3 animals offered the same diet for 29 or 30 days, and zinc-repleted for 3 and 12 hours, had serum levels of 82.5 μg/100 ml. By comparison, 3 rats fed a pair-fed control diet for 22 or 23 days showed serum concentrations of 123 μg/100 ml, and 3 animals on days 29 and 30 in this group had serum zinc levels of 192 μg/100 ml. Zinc concentrations of 163 μg/100 ml of serum were observed in 5 rats fed the ad libitum control diet for 7 to 17 days. These serum zinc concentrations represent pooled samples for the different groups of rats. Mean zinc concentrations of the testes are shown in figure 2. Although few rats were studied, there were marked differences between deficient and the pair-fed groups.

DISCUSSION

One of the objectives in this study was to determine which cells of the esophageal mucosa and in the testis are initially injured in zinc deficiency. In the normal rat esophagus, parenterally injected radioactive zinc concentrates in the mucosa of this organ (7), but the relative distribution of zinc in the different cell layers is not known. Some in vitro studies (8–10), using histologic sections of the skin, have shown that the granular layers in particular have a marked affinity for exogenous zinc. Whether there is a high concentration of zinc in these layers in vivo is not known. Interestingly, keratohyalin granules and tonofibrils in the granular cells have been considered as precursors of keratin (11). In view of these reports, we initially considered the probability that the primary locus of zinc action might be at the stage when the granular cells differentiate to fully keratinized cells of the stratum corneum. The results obtained, however, show that the cells immediately adjacent to the basal layer remain polyhedral in shape early in zinc deficiency. Furthermore, zinc repletion caused reversion of the morphology of the cells in this region to stellate-shaped ones before any change in the more superficial layers became evident (fig. 3 (3)). These stellate-shaped cells may either be previously polyhedral cells whose morphology has changed following zinc repletion, or newly formed cells from the basal layer. Thus, it is clear that the deepest layers of the transitional cells are injured in zinc deficiency, and it is possible that the subsequent abnormal differentiation to parakeratotic cells is a manifestation of this earlier injury.

Although keratohyalin granules were diminished in zinc deficiency and became more numerous following repletion, these findings are common in other diseases causing parakeratosis of the skin (12). The successive histologic changes following zinc repletion suggest that healing apparently occurs by displacement of the

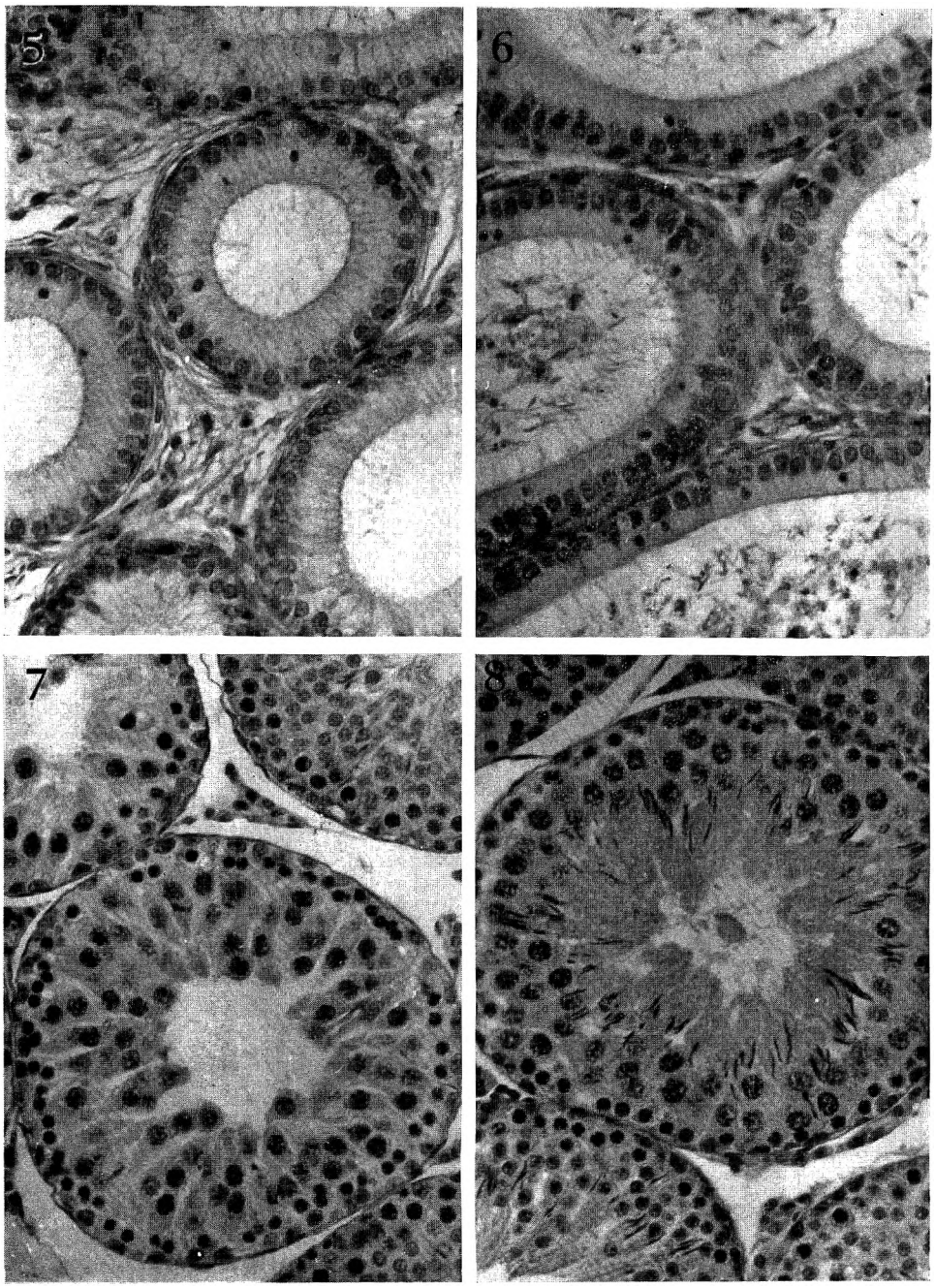

Fig. 4 (5–6) Junction of the caput-corpus in the epididymides of a zinc-deficient rat (5), and the pair-fed control (6) on day 23 of the experiment. Note the absence of sperm in the epididymal canal of the deficient rat. (7–8) Seminiferous tubules in stage 3 of Roosen-Runge's classification in a zinc-deficient rat (7) and the pair-fed control (8) on day 30. There is a reduction in number of elongated spermatids in the deficient rat.

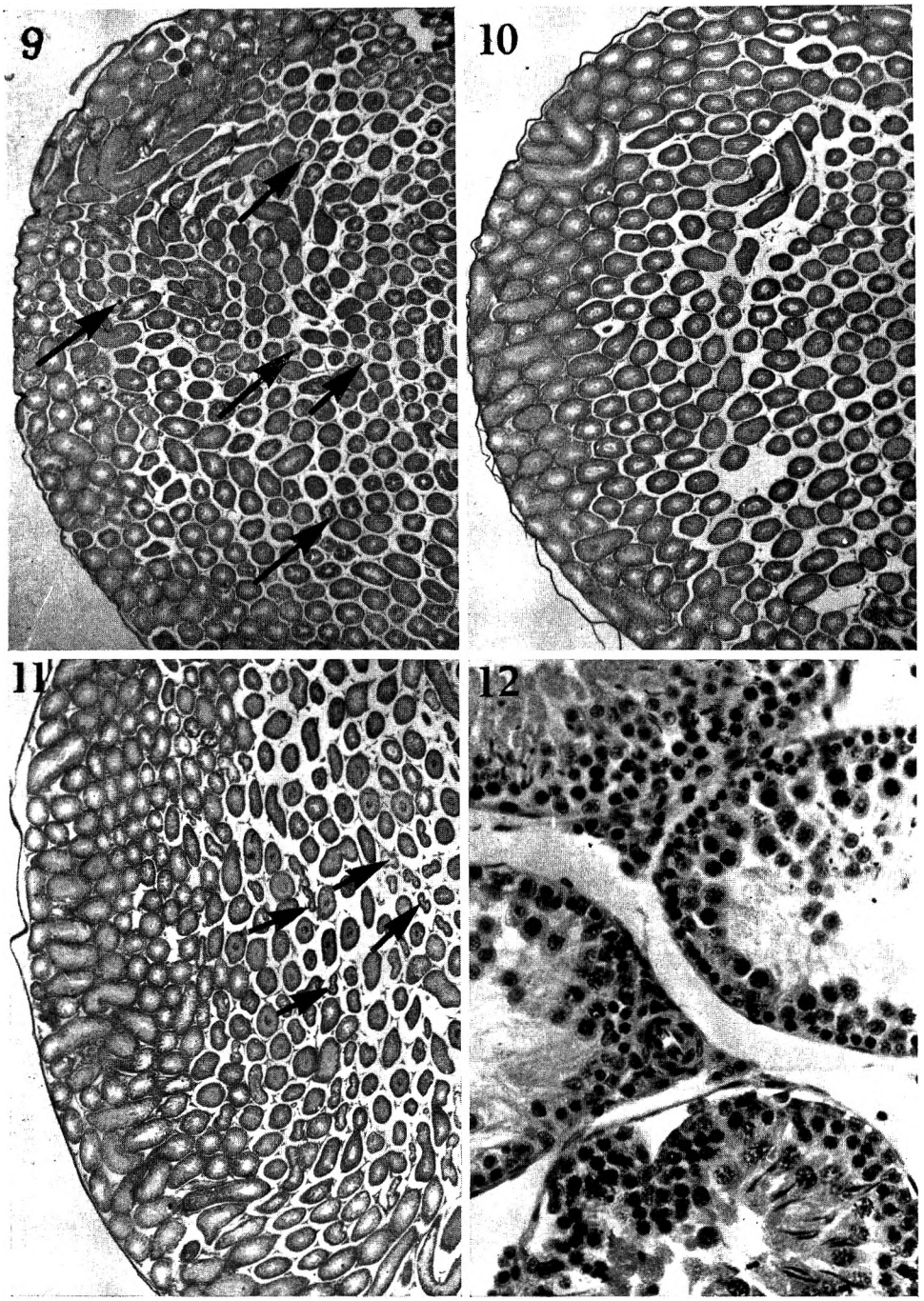

Fig. 5 (9–10) Testes of a zinc-deficient rat (9) and the pair-fed control (10) on day 30 of the experiment. The seminiferous tubules show early stages of degeneration (arrows) among healthy ones. (11) Other testis of the same rat repleted with zinc for 2 weeks in microphotograph 9. Arrows indicate atrophic tubules. (12) Same testes as microphotograph 11 with the 2 center tubules lined only with spermatogonia and spermatocytes.

abnormal cell layers above the stratum basal by normally differentiating cells that have newly arisen from this layer. In the normal esophageal mucosa some cells formed from the basal cells when displaced from their layer, differentiate irreversibly into stratum corneum cells (13). It is possible that in zinc deficiency, cells already derived from the basal layer prior to zinc repletion, also continue to differentiate into parakeratotic cells. It would be of interest in future studies to identify definitively the new cells from the basal layer following zinc repletion by concomitant labeling of their nuclei with parenterally injected thymidine.

In the esophagus of the normal rat, the basal layer appears to be the only site of the production of new cells (14). This characteristic appears preserved in zinc deficiency since we have not seen mitotic figures in the transitional cells, even though these cells were markedly increased in number.

The rapidity with which the esophageal lesions are reversed following zinc therapy is indeed remarkable. Such rapidity is conceivable since it takes only about 2 days for a basal cell to migrate from the basal layer and become keratinized in the normal rat esophagus (15).

In the testes of the zinc-deficient animals fed their diet for 23 days, there was a decreased number of spermatids with many seminiferous tubules appearing immature in comparison to those of the pair-fed controls. Thus, in zinc-deficiency of the rat the transformation of spermatids to spermatozoa is inhibited, and this appears to be the earliest histologic change in spermiogenesis. Nevertheless, in a recent study in 38-day-old rats previously fed a zinc-deficient diet for one week, there was biochemical evidence of increased breakdown of RNA and protein in the testes, although there were no apparent histologic changes (16). Since the tubules of the testes were still immature when examined, and spermiogenesis had not yet been fully established, the younger cells such as spermatocytes and spermatogonia are probably injured early in zinc deficiency despite the absence of demonstrable morphologic abnormality.

In the present study, it is of interest that despite zinc repletion, the histologic lesions appeared to have progressed further so that in many of the atrophic tubules only spermatocytes and spermatogonia remained. One explanation that could be offered for this finding is the possibility that cells already damaged before zinc repletion proceed to degenerate irreversibly, and are extruded and lost from the germinal epithelium. Conceivably, as in the case of the esophagus, reversal of the lesions in the testis following zinc repletion may occur by replacement of abnormal spermatogenic stages by new cells from the spermatogonia. Considering the fact that it takes about 45 days for spermatogonia in the normal rat to develop into mature sperm (17), the 2-week repletion period in the present study may have been too short for morphologic reversal of the lesions to become evident. Millar and co-workers (2) concluded from their earlier studies that the reversibility of the histologic lesions in the testes following zinc repletion depends on the severity of damage at the time of repletion. However, the testicular lesions produced by prolonged deficiency would be concurrent with the injurious effects of inanition, since anorexia develops quite early in zinc deficiency (3). Other workers (18) have demonstrated, by histochemical methods, decreased activities of certain enzymes in the zinc-deficient testis which activities are restored to normal following repletion with zinc.

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Tyrosine Toxicity in the Rat: Effect of high intake of p-hydroxyphenylpyruvic acid and of force-feeding high tyrosine diet [1]

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ABSTRACT Rats fed a low protein diet containing 5% of tyrosine develop external pathological lesions within a few days. The objective of the experiments described below was to determine whether this syndrome could be produced by feeding *p*-hydroxyphenylpyruvic acid or was altered by maintaining the food intake of rats fed a high tyrosine diet through force-feeding. When *p*-hydroxyphenylpyruvic acid was substituted for tyrosine, signs of toxicity did not develop within 2 weeks. Plasma tyrosine concentration and liver tyrosine transaminase activity were greatly elevated in rats fed the high tyrosine or the high *p*-hydroxyphenylpyruvic acid diet. The activity of *p*-hydroxyphenylpyruvate hydroxylase was not elevated in rats fed a high tyrosine diet; however, this enzyme appeared to be preferentially retained in the livers of rats fed *p*-hydroxyphenylpyruvic acid while liver weight was decreasing. Rats force-fed the high tyrosine diet showed only a transitory improvement in weight gain and 2 of 7 died within 8 days, which indicates that low food intake is an effect and not a cause of tyrosine toxicity. The results make it unlikely that intermediates of the main pathway of tyrosine degradation are responsible for the development of the signs of tyrosine toxicity.

Food intake and growth of rats fed a low protein diet containing an excessive amount of tyrosine are depressed, and the animals develop eye and paw lesions (1, 2). Also, food intake and growth of rats fed a low protein diet in which there is an amino acid imbalance are depressed; however, when the food intake of rats fed a diet in which there is an amino acid imbalance is stimulated by injecting them with insulin (3), by exposing them to a cold environment (4) or by force-feeding them (5), growth rate is improved and they appear normal. Since tyrosine toxicity is alleviated by supplementary threonine (2), it was thought that a dietary excess of tyrosine may increase the requirement for threonine and that the condition may be analogous in some respects to an amino acid imbalance. Thus the question arose as to whether tyrosine toxicity might be alleviated if the food intake of rats fed a high tyrosine diet were increased.

The observation that an excessive tyrosine intake increases tyrosine transaminase (L-tyrosine:2-oxoglutarate amino transferase, E.C. No. 2.6.1.5) activity (6), which should facilitate conversion of tyrosine to *p*-hydroxyphenylpyruvate, raised the question as to whether a high intake of *p*-hydroxyphenylpyruvic acid might cause signs of tyrosine toxicity.

The results presented here indicate that tyrosine toxicity is not alleviated when the food intake of rats fed a high tyrosine diet is increased by force-feeding them, and that intermediates of the main pathway of tyrosine catabolism are not responsible for the development of the toxicity syndrome.

METHODS

Male albino rats (Holtzman strain) weighing 50 to 55 g were individually housed in suspended screen-bottom cages and kept at a temperature of 24° in an animal laboratory with a light period from 8:00 AM to 8:00 PM. They were fed a basal 6% casein diet for 2 to 3 days, and

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were then separated into groups and fed the experimental diets for periods as indicated in the respective figures and tables. The diets contained 6% of casein, 5% of corn oil and adequate quantities of vitamins and minerals and were prepared as agar gels (2, 7). Substances added to the diet replaced an equivalent amount of the dietary carbohydrate. Food and water were given ad libitum except as indicated. Food intake was recorded daily and weight changes every other day.

In the force-feeding study, the basal diet was prepared in liquid form to contain approximately 0.8 g of dry matter per ml. For 2 days all rats were given 6 ml of this diet by stomach tube in 3 feedings per day at 9:00 AM, 3:00 PM and 9:00 PM to allow them a period of adjustment. Animals were then separated into 2 groups of 7 rats each, and fed the basal diet or a high tyrosine diet (5% L-tyrosine) in liquid form but the daily intake was divided into 2 meals, one at 9:00 AM and another at 9:00 PM. The 2 groups were tube-fed equal amounts of food during the experiment. The first day, they were given a total of 7 ml; this was increased to 8 ml on the second and third day. On days 4 to 6 the daily intake was increased to 9 ml and on days 7 and 8 to 9.5 ml per day. Concurrently a group of 4 rats was fed the high tyrosine diet ad libitum so that it would be possible to compare the growth of this group with that of the force-fed group.

Enzyme assay. At the end of experimental periods, rats were anesthetized with ether, blood was obtained by heart puncture with a heparinized syringe. The liver was excised and kept frozen at $-20°$ until assayed for liver enzymes. Twenty percent liver homogenates were prepared in 0.14 M potassium chloride solution which contained 0.005 M sodium hydroxide and were centrifuged at 25,000 $\times$ *g* for 45 minutes in a refrigerated centrifuge The activities of tyrosine transaminase and *p*-HPP-hydroxylase (*p*-hydroxyphenylpyruvate ascorbate:oxygen oxireductase, E.C. No. 1. 14.2.2) in the supernate were assayed at 30° using the enolborate methods (3).

Plasma amino acids. Plasma was separated from the red cells by centrifugation at 2,000 rpm for 15 minutes in a refrigerated centrifuge. The plasma proteins were precipitated by adding 15% sulfosalicylic acid solution to give a final concentration of 3%. The precipitate was removed by centrifugation at 25,000 $\times$ *g* for 20 minutes in a refrigerated centrifuge. Amino acid composition of the supernate was determined using a Technicon amino acid analyzer.

RESULTS

Effect of feeding p-hydroxyphenylpyruvic acid. Figure 1A shows that rats fed the 5% tyrosine diet ad libitum lost weight. Also, they all developed external pathological lesions and one rat died during the experiment. Food intake of these rats was severely depressed and plasma tyrosine concentration was greatly elevated (table 1). However, despite the slow growth rate of rats fed the diet containing 5% of *p*-hydroxyphenylpyruvic acid (*p*-HPPA), none of the animals developed external pathological lesions and all survived and looked healthy. Their food intake was somewhat above that of rats fed the high tyrosine diet, and plasma tyrosine concentration, although much lower than that of rats fed the high tyrosine diet, was greatly increased (table 1).

When the experiment was repeated for a 10-day feeding period, almost the same growth rates (fig. 1B) and food intake patterns were obtained (table 2) as in the previous experiment. Liver weight, as a percentage of body weight, was not affected by a high tyrosine intake, however, it was markedly decreased by a high intake of *p*-HPPA.

Values for tyrosine transaminase activity for rats fed the high tyrosine or high *p*-HPPA diets for 10 days were significantly higher than those for the control group. Values for *p*-HPP-hydroxylase activity for the 3 groups, expressed per 100 g of body weight, were not different; however, when expressed per gram of liver, the value for the rats fed the keto acid was significantly greater than those for the other 2 groups.

Plasma tyrosine concentration increased greatly in rats fed either the high tyrosine or the 5% *p*-HPPA diet for 10 days (table 3). The general decrease in plasma amino acids of rats fed the high tyrosine diet can

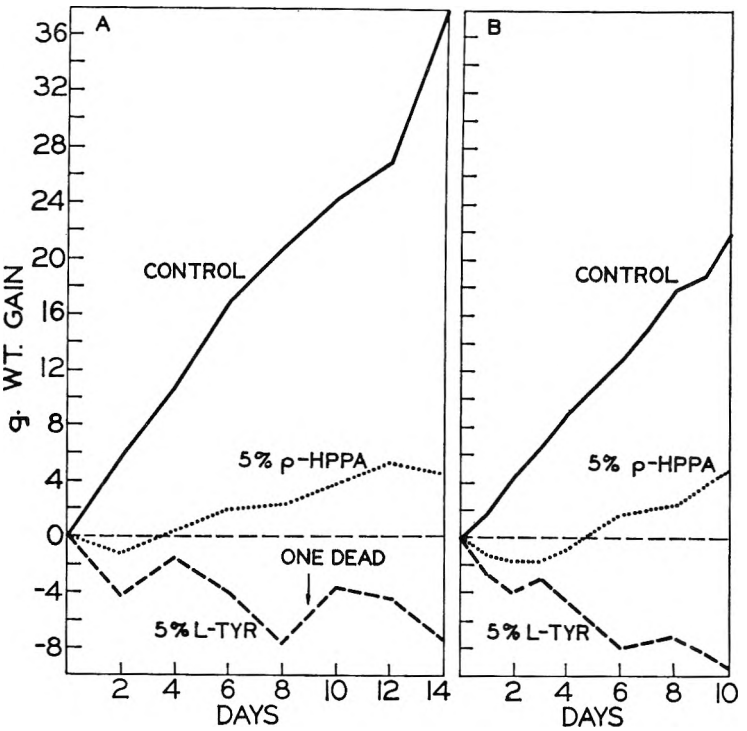

Fig. 1 Weight gain of young rats fed low protein diets containing 5% *p*-hydroxyphenylpyruvic acid (*p*-HPPA) or 5% tyrosine. (A) 2 weeks, and (B) 10-day experimental period with 4 rats/group.

TABLE 1

Effect of feeding young rats for 2 weeks diets containing 5% of L-*tyrosine or 5% of p-hydroxyphenylpyruvic acid (p-HPPA)*

Diet	No. showing signs of toxicity	Food consumed	Wt gain [1]	Plasma Tyr
		g	*g*	*μmole/100 ml*
Basal	0/4	135.2	37.8 ± 3	6.0
Basal + 5% L-Tyr	4/4	59.5	−7.4 ± 1	207.9
Basal + 5% *p*-HPPA	0/4	79.0	4.5 ± 1	66.6

[1] Mean ± SE.

TABLE 2

Effect of feeding young rats for 10 days diets containing 5% of p-hydroxyphenylpyruvic acid (p-HPPA) or 5% of L-*tyrosine*

	Food intake	Wt gain [1]	Liver wt [1]	Enzyme activity [2]/g liver		Enzyme activity [2]/100 g body wt	
				T.T.	*p*-HPPH	T.T.	*p*-HPPH
	g/10 days	*g/10 days*	*g/100 g body wt*				
Control	88.4	22 ± 3	5.9 ± 0.4	25.8 ± 2.9	56.3 ± 7.7	152 ± 17	333 ± 35
5% *p*-HPPA	52.9	5 ± 1	3.7 ± 0.2	65.7 ± 7.2 [3]	76.0 ± 4.4	248 ± 37 [4]	287 ± 26
5% L-Tyr	39.4	−9 ± 1	6.0 ± 0.1	74.9 ± 5.3 [3]	48.4 ± 7.3	447 ± 18 [3]	289 ± 38

[1] Mean ± SE.
[2] Tyrosine transaminase (T.T.) and *p*-hydroxyphenylpyruvate hydroxylase (*p*-HPPH) activities are expressed as μmole *p*-hydroxyphenylpyruvic acid formed or consumed/hour, respectively.
[3] Significantly different from control $P < 0.01$.
[4] Significantly different from control $P < 0.05$.

TABLE 3

Effect of feeding 5% p-hydroxyphenylpyruvic acid (p-HPPA) or 5% L-Tyr for 10 days on plasma amino acids

Amino acid	Plasma amino acid concn [1]		
	Control	5% L-Tyr	5% p-HPPA
	μmole/100 ml	*μmole/100 ml*	
Ser	35.2	24.0	39.9
Glu	12.0	8.1	11.5
Gly	18.1	15.0	21.7
Ala	99.8	53.8	82.9
Val	16.6	9.9	10.9
Ile	7.0	3.9	5.1
Leu	12.5	7.1	8.4
Tyr	8.7	170.0	113.0
Phe	7.9	3.9	6.5
Lys	56.7	45.9	64.1
His	12.3	5.7	13.1
Arg	9.6	8.3	10.0

[1] Pooled plasma from 4 rats/group.

probably be attributed to their low food intake. Branched-chain amino acid concentrations were low in plasma of rats fed the 5% *p*-HPPA diet but the concentrations of other amino acids were similar to the control values.

Effect of force-feeding 5% L-tyrosine diet. Figure 2 shows that while rats fed the high tyrosine diet ad libitum lost weight, the group force-fed this diet gained but not as much as the group force-fed the basal diet. No rats force-fed the basal diet died during the 8-day experimental period. Two of the seven force-fed the high tyrosine diet died but not after a prolonged period of debilitation as observed with rats fed this diet ad libitum. They developed only mild signs of tyrosine toxicity and died about 24 hours later. As they died several hours after being fed, their deaths cannot be attributed to food being forced into their lungs.

Force-feeding the high tyrosine diet caused a fourfold increase in tyrosine transaminase activity whether expressed per unit of liver weight or per unit of body weight (table 4). However, *p*-HPP-hydroxylase activity was not significantly affected.

DISCUSSION

Although growth rate and food intake of rats fed the low protein diet containing 5% of *p*-hydroxyphenylpyruvic acid were depressed, the animals did not develop eye and paw lesions characteristic of tyrosine

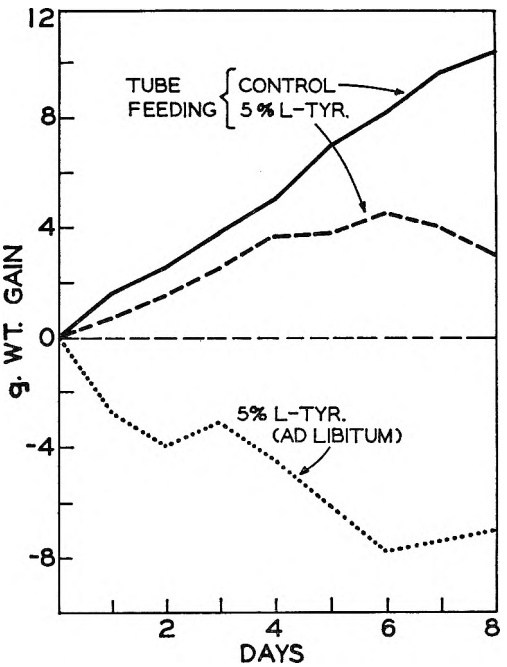

Fig. 2 Weight gain of rats force-fed a high tyrosine diet.

toxicity (1, 2, 9). Lesions develop within a few days in rats fed ad libitum a diet containing 5% of L-tyrosine. It is, therefore, unlikely that *p*-hydroxyphenylpyruvic acid or other intermediates on the main pathway of oxidation of tyrosine to carbon dioxide are responsible for the toxicity syndrome that develops as a result of an excessive intake of tyrosine.

Enzyme activities per 100 g of body weight are indicative of the total degradative capacity of the animal and are therefore considered to be the most meaningful basis for comparison in these studies. Comparison of the enzyme values per gram of liver with those per 100 g body weight indicates that the decrease in liver weight of rats fed the keto acid was accompanied by preferential retention of *p*-HPP-hydroxylase. Zannoni and LaDu (9) injected guinea pigs intraperitoneally with *p*-HPP and observed no change in *p*-HPP-hydroxylase activity 1 hour later.

The ready reversibility of the tyrosine transaminase reaction is evident from the increased plasma tyrosine concentration of rats fed *p*-HPPA. It is also evident that, despite its preferential retention, *p*-HPP-

TABLE 4

Effect of force-feeding a 6% casein diet containing 5% L-tyrosine for 8 days

	Liver wt	Enzyme activity [1]/g liver		Enzyme activity [1]/100 g body wt	
		T.T.	*p*-HPPH	T.T.	*p*-HPPH
	g/100 g body wt				
Control [2]	4.39 ± 0.1	20.2 ± 2.3	75.7 ± 13.9	88 ± 9.3	332 ± 54
5% L-Tyr [3]	4.71 ± 0.7	85.2 ± 15 [4]	60.1 ± 7.6	400 ± 67 [4]	281 ± 32

[1] Tyrosine transaminase (T.T.) and *p*-hydroxyphenylpyruvate hydroxylase (*p*-HPPH) activities are expressed as μmole *p*-hydroxyphenylpyruvic acid formed or consumed/hour, respectively.
[2] Mean ± SE for 6 rats.
[3] Mean ± SE for 4 rats.
[4] Significantly different from control $P < 0.01$.

hydroxylase activity is not high enough to permit rapid degradation of the load of *p*-HPPA ingested by these animals. A high intake of tyrosine causes induction of tyrosine transaminase (6), and hence, presumably, the high plasma tyrosine concentration is responsible for the elevated tyrosine transaminase activity of rats fed an excessive amount of *p*-HPPA.

Rats force-fed the high tyrosine diet grew more rapidly than those fed this diet ad libitum but not as rapidly as those fed the basal diet; hence, growth failure of rats fed a high tyrosine diet ad libitum is not due solely to depressed voluntary food intake. Also, although rats that were force-fed the high tyrosine diet did not develop as severe lesions of tyrosine toxicity, they developed mild lesions and some died earlier than those fed ad libitum.

Development of the toxicity syndrome is associated with high tissue tyrosine concentrations. In rats fed diets containing 3% or 5% of L-tyrosine and showing signs of toxicity, plasma tyrosine concentrations are usually between 150 and 250 μmoles/100 ml of plasma (10). With diets containing 3% of L-tyrosine, values of between 100 and 150 μmoles/100 ml are observed occasionally but with this dietary tyrosine content signs of toxicity usually occur in only half to two-thirds of the animals (2). Also in rats receiving high tyrosine diets containing supplements of threonine plasma tyrosine concentrations of 77 to 110 μmoles/100 ml are observed without signs of tyrosine toxicity developing. The values of 67 and 113 μmoles/100 ml in the present study are in this latter range. This raises a question as to whether signs of tyrosine toxicity might be induced by feeding larger quantities of *p*-HPPA, particularly if blood tyrosine concentration were elevated somewhat more.

Whether tyrosine itself or some product other than products on the main pathway of tyrosine oxidation is the toxic agent has not been established. It is of interest in relation to this that administration of thyroxine, itself a product of tyrosine metabolism, increases the severity of the signs of tyrosine toxicity.[3]

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Distribution and Excretion of Nickel-63 Administered Intravenously to Rats

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ABSTRACT The distribution and excretion of ^{63}Ni administered intravenously was studied using 64 Sprague-Dawley female rats. Small quantities of tissues (50–75 mg) were acid-digested and the isotope was counted using a liquid scintillation system. The major portion (61%) of a single intravenous injection of ^{63}Ni was excreted via the urine within 72 hours, with a lesser amount appearing in the feces (5.9%). In addition, the ^{63}Ni activity in the whole blood and plasma had completely disappeared by 48 hours with no accumulation in the red blood cells. The relative distribution of the ^{63}Ni per gram of fresh tissue was as follows: kidney > adrenal > ovary > lung > heart > eye > thymus > pancreas > spleen > liver > epidermis > gastrointestinal tract > muscle > incisor > femur = brain > adipose. However, in all tissues analyzed the ^{63}Ni decreased rapidly, and after 72 hours only the kidney contained significant amounts of ^{63}Ni. The ^{63}Ni distribution was compared with the blood volume of specific tissues. Correlation coefficients were 0.79, 0.76, 0.82 and 0.68 for intervals of 15 minutes, 2 hours, 6 hours, and 16 hours, respectively, following intravenous injection of the isotope. These correlations were statistically significant and suggest that the distribution of the ^{63}Ni was directly dependent upon the relative blood volume of the specific tissues analyzed.

This laboratory has initiated a program to investigate the importance of various trace elements heretofore not shown to be essential to life. Nickel was one of the elements chosen to be studied first for the following reasons. It consistently occurs in plant and animal tissues including the newborn (1). It is reported to activate various enzyme systems including arginase (2) carboxylase (3) acetyl coenzyme A synthetase (4) and trypsin (5). Nickel is present in RNA from various sources (6) and may play a part in maintaining the configuration of the protein molecules of crystalline complexes of ribonuclease (7). Finally, this element is of clinical interest since it is reported to be greatly elevated in serums from patients with myocardial infarction (8) and has been implicated as a pulmonary carcinogen in tobacco smoke (9). However, few studies have dealt with the retention or excretion of nickel in man or animals. The only investigation found that used radioactive nickel (^{63}Ni) was that by Wase et al. (10) in 1954, who studied the distribution and excretion of ^{63}Ni using a high level, 102 ug ^{63}Ni/mouse, administered intraperitoneally. The purpose of the present paper is to report the distribution and excretion of ^{63}Ni given intravenously to rats in trace amounts. A simple method for sample preparation and counting ^{63}Ni in biological samples with the use of liquid scintillation is also described.

EXPERIMENTAL

The sample preparation and counting procedure adapts the method described by Mahin and Lofbert (11) for counting tritium ^{14}C and ^{35}S in biological tissue. Briefly, the method uses acid digestion of small quantities of tissues at a low temperature. Up to 70 mg of fresh tissue or 200 mg of blood or urine were digested directly in the scintillation counting vials using 0.2 ml of 70% perchloric acid and 0.4 ml of 30% hydrogen peroxide. The vials were tightly capped and heated at 70° in a drying oven until the solution cleared, indicating that oxidation was complete. After cooling, 6 ml of ethylene glycol monethyl ether (Cellosolve) were added. In addition, 10 ml of the toluene [1] phosphor solution containing 6 g of 2,5 diphenyloxa-

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[1] Spectroquality reagent grade toluene. Matheson Coleman and Bell, East Rutherford, New Jersey.

zole (PPO)/liter of toluene were added. Before counting, the samples were allowed to equilibrate for 2 hours. For counting, a Beckman liquid scintillation counter was used with an adjustable iso-set module. The lower window was closed, with the upper window reading 3.5. The gain was adjusted to give an external standard counting ratio of 1.300 for the least quenched sample. A quenching curve was prepared using 1 μCi ^{63}Ni treated in the same manner as described for the tissue, with the exception that increasing quantities of bromcresol green indicator were added to the 2 vials after heating to give varying intensities of yellow color. The resulting quench curve (external standard ratio plotted against counting efficiency) was a straight line. Counting efficiency was 42% for the least quenched samples. All counts were adjusted to an external standard ratio of 1.300. Samples were counted to a 3% statistical counting error.

Radioactive nickel as $^{63}NiCl_2$ [2] in 1 N HCl was diluted with normal saline to give a stock solution of 10 μCi ^{63}Ni/ml. Either 0.25 ml or 0.5 ml of this stock solution was injected per animal, representing 2.5 μCi or 5 μCi of ^{63}Ni. Since the specific activity of the nickel was 3.4 mCi/mg the doses were equivalent to 0.74 μg or 1.47 μg ^{63}Ni.

Female rats of the Sprague-Dawley strain averaging 218 ± 26 g were used. They were fed laboratory ration [3] and tap water ad libitum.

For the excretion studies 5 rats were maintained in individual plastic metabolism cages.[4] The animals were adapted to the cage for 3 days before injection of the isotope. Each animal received 5 μCi of ^{63}Ni intravenously via the saphenous vein after light anesthesia with ether. Total urinary and fecal output were collected at intervals of 2, 4, 6, 8, 24, 48 and 72 hours.

The distribution studies were divided into 2 experiments. Experiment 1 used 5 animals per group which were intravenously injected with 2.5 μCi ^{63}Ni. They were held in individual suspended wire cages and killed at intervals of 0.25, 1, 2, 4, 8, 16, 24, 48 and 72 hours. After the proper interval the animals were exsanguinated under ether anesthesia by heart puncture to remove as much blood as possible from the tissues. Samples to be analyzed were excised, weighed, and frozen. Before analysis the tissues were minced and well-mixed so that a representative aliquot could be obtained. The samples for counting analyses in experiment 1 included whole blood, plasma, kidney, lung, spleen, liver and femur.

In experiment 2, four animals were used per group, injected similarly with 5.0 μCi ^{63}Ni. They were killed at 0.25, 2, 6, 16 and 72 hours. In addition to those samples analyzed in experiment 1, the following specimens were studied for ^{63}Ni distribution: blood cells, adrenal, ovary, heart, eye, thymus, pancreas, skin, brain, adipose, muscle, incisor and gastrointestinal tract. The carcasses of those animals killed at 72 hours were digested and an aliquot was counted for ^{63}Ni activity. Before the blood cells were counted, they were washed to remove plasma. This was accomplished by adding physiological saline (2 times the volume of cells) to the cells, mixing well, centrifuging at 2000 rpm for 10 minutes and decanting the saline. This procedure was repeated 3 times. Both the diet and drinking water were analyzed for nickel by atomic absorption spectrophotometry.[5] The diet was ashed by heating to 550° for 18 hours. Minimal quantities of concentrated hydrochloric acid were added. The reference standards for the diet analysis contained hydrochloric acid as the diluent. The drinking water (tap) was analyzed after concentrating 50-fold by boiling. Deionized water (nickel level non-detectable) was used as the solvent for the reference standards.

RESULTS

The accumulated excretion rate of ^{63}Ni via the urine and feces is shown in figure 1. Over 60% of the injected dose of ^{63}Ni was excreted by the urine within 72 hours. In contrast, less than 6% was found in the feces for the same duration. The data are presented as hourly urinary (fig. 2) and fecal (fig. 3) excretion of ^{63}Ni. The results show that the excretion rate was highest for the urine 2 hours following in-

[2] New England Nuclear Corporation, Boston.
[3] Lab Blox, Allied Mills, Inc., Chicago.
[4] Model 110, Maryland Plastics, Inc., 9 East 37th Street, New York.
[5] Model 303, Perkin-Elmer Corporation, Norwalk, Connecticut.

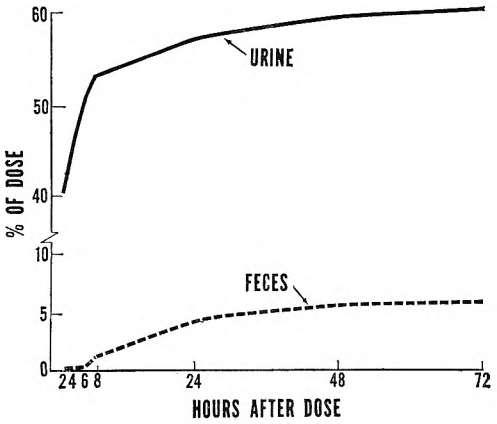

Fig. 1 Accumulated excretion of ^{63}Ni via the urine and feces following a single intravenous dose.

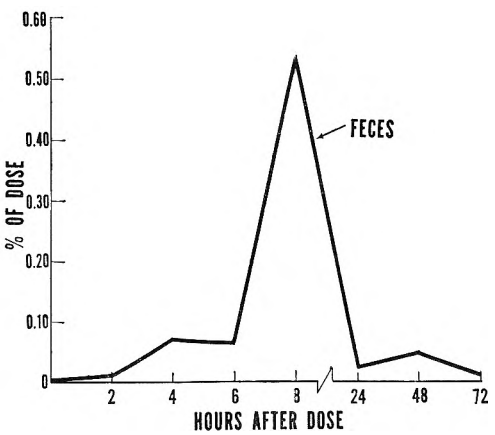

Fig. 3 Average hourly fecal excretion of ^{63}Ni following a single intravenous dose.

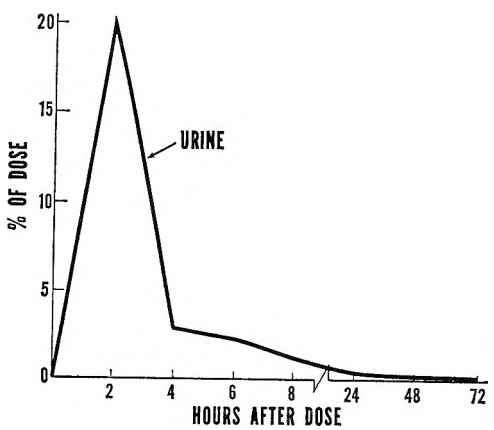

Fig. 2 Average hourly urinary excretion of ^{63}Ni following a single intravenous dose.

jection. In contrast, fecal excretion did not peak until 8 hours.

The results of the distribution study of experiment 1 are presented in table 1. The amount of ^{63}Ni retained per gram of tissue varied widely among the tissues analyzed. However, with the exception of the kidney, no significant amount of activity remained after 72 hours. In addition, by 48 hours all activity had disappeared from the whole blood and plasma (fig. 4).

The results of the more complete distribution study (exp. 2) are given in table 2. The greatest retention rate again occurred in the kidney. At 15 minutes after injection, the distribution of ^{63}Ni was as follows: kidney > adrenal > ovary > lung > heart > eye > thymus > pancreas >

TABLE 1

Distribution of ^{63}Ni in selected tissues at timed intervals after single intravenous dose [1]

Time after dose	Kidney	Lung	Spleen	Liver	Femur
hours			*% dose/g fresh tissue*		
0.25	6.54 ± 0.50	0.97 ± 0.06	0.40 ± 0.30	0.36 ± 0.03	0.24 ± 0.02
1	5.47 ± 0.38	0.66 ± 0.03	0.22 ± 0.01	0.24 ± 0.01	0.16 ± 0.01
2	4.46 ± 0.36	0.42 ± 0.03	0.14 ± 0.01	0.14 ± 0.01	0.08 ± 0.01
4	2.36 ± 0.16	0.32 ± 0.01	0.10 ± 0.00	0.12 ± 0.01	0.04 ± 0.00
8	1.57 ± 0.16	0.18 ± 0.01	0.05 ± 0.00	0.06 ± 0.01	0.01 ± 0.00
16	0.95 ± 0.06	0.11 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	nd [2]
24	0.92 ± 0.11 [3]	0.11 ± 0.01 [3]	0.07 ± 0.01 [3]	0.02 ± 0.00 [3]	nd [3]
48	0.52 ± 0.04	0.03 ± 0.00	0.01 ± 0.00	nd	0.01 ± 0.00
72	0.27 ± 0.02	0.02 ± 0.00	0.01 ± 0.00	nd	0.01 ± 0.00

[1] Mean ± SE, 5 animals/group.
[2] Non-detectable.
[3] Four animals used for 24-hour interval.

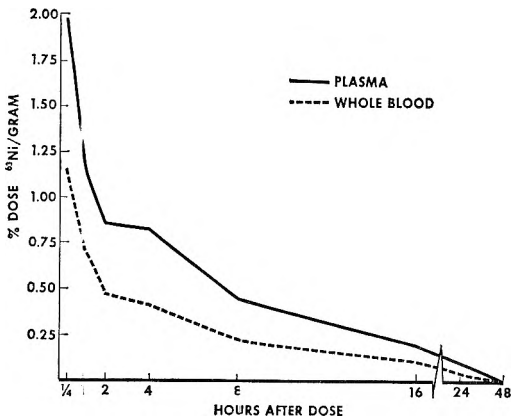

Fig. 4 Disappearance of ^{63}Ni from blood and plasma after single intravenous dose.

spleen > liver > epidermis > gastrointestinal tract with contents > muscle > incisor > femur = brain > adipose. In all specimens the ^{63}Ni decreased rapidly. As in experiment 1, 72 hours after injection only the kidney contained significant amounts of ^{63}Ni. The disappearance of the isotope from whole blood and plasma agreed closely with the results of experiment 1 with no activity present after 72 hours. In addition, no significant amount of ^{63}Ni activity was found in the blood cellular components when they were counted directly in this experiment.

DISCUSSION

The literature has been contradictory concerning nickel excretion. For example Drinker et al. (12) reported in 1924 that in human subjects using nickel cooking utensils, the majority of ingested nickel was excreted in the feces with little in the urine. In contrast, Kent and McCance (13) observed more nickel excreted in the urine than the feces, using 2 men. When intravenous nickel was given, the output of nickel increased in the urine but not in the feces. Using mice injected intraperitoneally, Wase et al. (10) noted the greatest excretion of ^{63}Ni in the feces. In a balance study on 4 dogs, Tedeschi and Sunderman (14) reported 90% of dietary ingested nickel excreted via the feces and 6% in the urine. However, following inhalation of nickel carbonyl, there was a sharp increase in urinary nickel, with fecal nickel remaining constant. The latter study and the experiments of Kent and McCance (13) indicate clearly that the method of administration directly affects the route of excretion for nickel. That is, using the data of Tedeschi and Sunderman (14), no more than 10% of ingested

TABLE 2

Distribution of ^{63}Ni in selected tissues at time-intervals after single intravenous dose [1]

	Hours after dose				
	0.25	2	6	16	72
	% dose/g fresh tissue				
Kidney	2.49 ± 0.54	2.57 ± 1.24	0.59 ± 0.23	0.20 ± 0.06	0.11 ± 0.06
Adrenal	0.92 ± 0.14	0.28 ± 0.03	0.15 ± 0.01	0.12 ± 0.02	0.03 ± 0.01
Ovary	0.90 ± 0.33	0.23 ± 0.06	0.11 ± 0.02	0.09 ± 0.01	nd [2]
Lung	0.81 ± 0.04	0.29 ± 0.04	0.14 ± 0.03	0.09 ± 0.01	0.01 ± 0.00
Heart	0.64 ± 0.04	0.21 ± 0.02	0.11 ± 0.01	0.07 ± 0.01	nd
Eye	0.56 ± 0.06	0.19 ± 0.03	0.13 ± 0.04	0.08 ± 0.03	0.01 ± 0.00
Thymus	0.55 ± 0.03	0.15 ± 0.02	0.12 ± 0.02	0.04 ± 0.01	nd
Pancreas	0.54 ± 0.05	0.16 ± 0.03	0.12 ± 0.03	0.08 ± 0.01	nd
Spleen	0.48 ± 0.03	0.13 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.01 ± 0.00
Liver	0.40 ± 0.03	0.13 ± 0.02	0.08 ± 0.01	0.05 ± 0.01	nd
Skin	0.38 ± 0.08	0.20 ± 0.05	0.07 ± 0.01	0.04 ± 0.01	nd
G.I tract	0.33 ± 0.04	0.21 ± 0.04	0.11 ± 0.03	0.10 ± 0.01	nd
Muscle	0.29 ± 0.07	0.11 ± 0.05	0.04 ± 0.01	0.03 ± 0.05	nd
Teeth	0.21 ± 0.04	0.08 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
Femur	0.15 ± 0.02	0.05 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	nd
Brain	0.15 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	nd
Adipose	0.14 ± 0.04	0.04 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	nd
Carcass	—	—	—	—	nd

[1] Mean ± SE, four animals/group.
[2] Non-detectable.

nickel is absorbed. Therefore the majority of orally administered nickel would be expected to appear in the feces. However, our data show that *intravenous* nickel is cleared quickly via the urine, with only small quantities occurring in the feces (figs. 1, 2 and 3). As a result of the role of the kidney in nickel clearance, the high radioactivity of ^{63}Ni in this organ is probably due to urine retained within the organ.

In experiment 1, it appeared that all of the radioactivity of whole blood could be accounted for by the plasma fraction. That is, the activity of the plasma portion of one gram of blood was equal to the total activity for a similar quantity of whole blood. By counting the cellular fraction directly, it was confirmed in experiment 2 that the blood cells did not contain significant ^{63}Ni. Thus, these data are in disagreement with the studies of Wase et al. (10) who found that the red blood cells contained approximately one-half the activity of plasma in mice. However, during the duration of our experiment, ^{63}Ni did not accumulate in the blood cells as has been reported for zinc (15), selenium (16) and chromium (17).

The relatively high activity of both the adrenal and ovary is of interest since these organs are controlled hormonally. However, nickel was not actively retained. No previous studies were found in the literature regarding normal concentration of nickel (radioactivity or non-radioactive) for these tissues.

In experiment 1, of the tissues analyzed, the lung had the second highest ^{63}Ni per gram of tissue and in experiment 2, it ranked fourth, exceeded only by the kidney, adrenal, and ovary. In the study of Wase et al. (10), the lung had the greatest retention of ^{63}Ni when compared with the other organs analyzed. The authors suggested a high complex constant for Ni^{++} with lung protein. In addition, Schroeder et al. (1) reported that this element occurred more frequently in the lung of humans than in the kidney, heart, larynx, bone, trachea or aorta.

Since the isotope quickly disappeared from both the blood and organs, it was reasoned that the distribution of ^{63}Ni in the various tissues studied may be directly related to the blood volume of these tissues, i.e., blood volume as microliters per gram of tissue. Everett et al. (18) have reported the blood volume of various tissue and organs in female Sprague-Dawley rats of a weight range similar to that used in our study. Therefore, using the data of Everett et al. (18), the relative blood volume and distribution of ^{63}Ni in various organs and tissue were compared (table 3). As shown, a high correlation existed between ^{63}Ni distribution and tissue blood volume at 15 minutes, 2, 6, and 16 hours following a

TABLE 3

Comparison of ^{63}Ni distribution with tissue blood volume

Tissue [1]	^{63}Ni distribution [2]				Blood vol [3]
	15 min	2 hr	6 hr	16 hr	
	% dose/g fresh tissue				*μliters/g*
Adrenal	0.92	0.28	0.15	0.12	268
Ovary	0.90	0.23	0.11	0.09	268
Lung	0.81	0.29	0.14	0.09	505
Heart	0.64	0.21	0.11	0.07	238
Spleen	0.48	0.13	0.11	0.09	134
Liver	0.40	0.13	0.08	0.05	265
Skin	0.38	0.20	0.07	0.04	18.2
Muscle	0.29	0.11	0.04	0.03	22.9
Femur	0.15	0.05	0.03	0.01	32.8
Brain	0.15	0.04	0.04	0.05	30.1
Correlation coefficient	0.79	0.76	0.82	0.68	
	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.05$	

[1] Kidney was omitted due to radioactive urine within it.
[2] No comparison could be made for the 72-hour interval because ^{63}Ni was non-detectable in several tissues (see table 2).
[3] Blood volume data from Everett et al. (18).

single injection of ^{63}Ni. These data strongly suggest that the distribution of ^{63}Ni was directly related to blood volume of the specific organ or tissue studied.

Because nickel has been reported to be present in ribonucleic acid (RNA) from various sources (6, 19), the level of ^{63}Ni was compared with the concentration of RNA in selected tissues. The RNA concentrations reported by Winick (20) for rat lung, heart, liver and spleen showed no significant correlation with the distribution of ^{63}Ni in these organs.

It is recognized that the animals in this experiment were not receiving a diet devoid of nickel. That is, analysis of the laboratory ration by atomic absorption spectrophotometry showed that the diet contained 3.3 μg of Ni/g. In addition, 2 μg of Ni/liter was found in the drinking (tap) water. Should a nickel-free diet be fed, it is conceivable that the excretion and distribution of injected ^{63}Ni would be quite different than reported here. Specifically, the injected ^{63}Ni of our experiments may be handled by the animal as excess nickel since an adequate quantity was being supplied by the diet. Current investigations to develop a nickel-deficient diet are in progress.

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Effect of Arginine upon the Toxicity of Excesses of Single Amino Acids in Chicks [1]

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ABSTRACT A series of experiments was conducted to elucidate the mechanism through which arginine relieves the imbalance caused by the addition of excessive levels of amino acids in chick diets. Excess quantities of the L-isomer amino acids, lysine, tyrosine, histidine, glycine, methionine, cystine, valine, threonine, isoleucine, phenylalanine, tryptophan, glutamic acid and leucine were added singly to a balanced low protein casein diet and fed to week-old chicks for 2 weeks' duration. Each of the amino acids fed at super-optional levels resulted in some manifestation of imbalance, i.e., reduction in growth or feed intake, or both, on the part of the chicks. Amounts of these amino acids required to bring about an imbalance, ranged from 10% (L-glutamic acid) to 0.4% (L-lysine·HCl). The aforementioned conditions of imbalance were, with the exception of those caused by tryptophan, leucine and glutamic acid, all alleviated to some extent by the addition of arginine. The level of arginine required to bring about this relief ranged from 0.3% to 2.0% but did not appear to be dependent upon the severity of the imbalance. Attempts to define the unique role of arginine in the detoxification of amino acid excesses were not successful. It appears that supplemental arginine in imbalanced diets does not exert its benefit because of a deficiency of this amino acid due to a reduced feed intake, does not mediate its influence through competitive inhibition at absorption or reabsorption sites, nor does it appear to act as a cycle intermediate in a possible latent urea cycle.

It is generally accepted that most amino acids, when offered in the diet greatly in excess of the requirement of an animal, will produce ill effects such as reduced feed intake and growth depression (1). It has also been shown that excess essential amino acids in chick diets can increase the chicks' requirement for certain other essential amino acids (2–5).

Among the most notable relationships demonstrated is the very much higher requirement for arginine of chicks fed a diet containing excess lysine. This relationship has been interpreted by some (6–8) as a specific antagonism between arginine and lysine. Boorman and Fisher (9), however, concluded that the lysine-arginine interaction is a nonspecific manifestation of a general phenomenon of amino acid detoxification.

Attempts to illustrate the effects of single amino acid excesses on chick performance have been confounded by the use of DL- isomers of amino acids (10) and by use of intraperitoneal injection of amino acids in which case gastrointestinal absorption is bypassed (11). Therefore, in the present study diets containing excess L- amino acids were fed under conventional conditions until toxic effects were noted. The effect of arginine on these toxicities was then studied.

Three possible mechanisms are offered to explain the role of arginine in situations of amino acid imbalance; (a) excess amino acids bring about a reduction in feed intake resulting in a deficiency of arginine for protein synthesis; (b) arginine competes with the amino acid in excess at the absorption site (gastrointestinal tract) so that excess quantities are not absorbed or at the reabsorption site (kidneys) so that reabsorption of the excreted excess amino acids does not occur, or under both of these conditions. This hypothesis is based on the findings of Rosenberg et al. (12) which indicate a mutual competitive inhibition between L-arginine, L-lysine and L-ornithine in the rat kidney; and (c) arginine acts at the metabolic

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level by increasing the efficiency of either or both the uric acid cycle or a latent urea cycle in the chicken by providing required cycle intermediates (5).

Within the framework of these hypotheses it is the purpose of this report to further demonstrate that a general detoxification mechanism involving arginine does exist for excess amino acids and to stimulate interest in the elucidation of this mechanism.

EXPERIMENTAL

The experiments described in this report all followed the same general plan. A basal diet containing casein to provide 12% crude protein was supplemented with histidine, glycine, arginine and methionine as shown in table 1. At this level of casein protein the chicks' requirement for arginine and methionine are just met by the supplementary levels indicated.[3] As a consequence, voluntary feed intake is maximized. Additions of other amino

TABLE 1

Basal diet containing 12.0% crude protein from casein [1]

	%
Glucose [2]	var
Casein [3]	14.679
Minerals [4]	5.270
Corn oil	1.000
Choline chloride	0.200
L-Histidine·HCl·H_2O	0.040
Glycine	2.093
L-Arginine·HCl	0.408
DL-Methionine	0.384
Vitamins (2.0 g/kg) [4]	+
Penicillin (11 mg/kg)	+
	100.00

[1] At this level of casein protein, the chicks' requirement for arginine and methionine is just met by the supplementary levels indicated and feed intake is maximized.

[2] Cerelose, the Canada Starch Company Limited, Cardinal, Ontario.

[3] Casein B-3-F, The Borden Chemical Company (Canada) Limited, West Hill, Ontario.

[4] Klain et al. (15).

[3] Unpublished data, University of Illinois, 1962.

TABLE 2

Chick performance with diets containing amino acid excesses [1]

Exp. no.	Amino acid added to basal diet [2]		Gain [3]	Feed consumption
	%	%	*% of basal*	*% of basal*
1	Lys	Arg		
	—	—	100[a] (205 g) [4]	100 (375 g)
	0.4	—	57[c]	78
	0.4	0.1	80[b]	87
	0.4	0.2	91[ab]	92
	0.4	0.3	95[a]	95
	0.4	0.4	89[ab]	95
	0.4	0.5	96[a]	92
	0.4	0.6	92[ab]	92
2	Tyr	Arg		
	—	—	100[a] (163 g)	100 (304 g)
	3.0	—	74[c]	84
	3.0	0.3	84[bc]	87
	3.0	0.6	90[ab]	88
	3.0	0.9	95[ab]	95
	3.0	1.2	82[bc]	90
	3.0	1.5	85[bc]	88
	3.0	1.8	82[bc]	89
3	His	Arg		
	—	—	100[a] (195 g)	100 (369 g)
	1.6	—	64[c]	73
	1.6	0.3	82[b]	86
	1.6	0.6	84[b]	83
	1.6	0.9	90[ab]	85
	1.6	1.2	89[ab]	86
	1.6	1.5	88[ab]	85
	1.6	1.8	86[b]	82

TABLE 2 (*Continued*)

Chick performance with diets containing amino acid excesses [1]

Exp. no.	Amino acid added to basal diet [2]				Gain [3]	Feed consumption
	%	%	%	%	*% of basal*	*% of basal*
4		Gly	Arg			
		—	—		100[a] (188 g)	100 (349 g)
		3.0	—		64[d]	64
		3.0	0.5		68[cd]	64
		3.0	1.0		73[bcd]	69
		3.0	1.5		78[bc]	74
		3.0	2.0		80[b]	76
		3.0	2.5		74[bcd]	72
		3.0	3.0		79[b]	76
5		Met	Arg			
		—	—		100[a] (194 g)	100 (344 g)
		1.0	—		69[d]	75
		1.0	0.3		78[bc]	80
		1.0	0.6		81[b]	83
		1.0	0.9		76[bcd]	75
		1.0	1.2		73[bcd]	76
		1.0	1.5		79[bc]	79
		1.0	1.8		72[cd]	73
6		Cys	Arg			
		—	—		100[a] (196 g)	100 (328 g)
		1.0	—		77[c]	87
		1.0	0.30		88[b]	87
		1.0	0.45		88[b]	88
		1.0	0.60		86[bc]	86
		1.0	0.75		85[bc]	83
		1.0	0.90		87[bc]	85
		1.0	1.05		86[bc]	84
7	Val	Thr	Il	Arg		
	—	—	—	—	100[ab] (193 g)	100 (363 g)
	2.0	—	—	—	87[b]	93
	—	2.0	—	—	69[c]	68
	—	—	2.0	—	86[b]	86
	—	—	—	0.6	103[ab]	94
	2.0	—	—	0.6	106[a]	97
	—	2.0	—	0.6	92[ab]	73
	—	—	2.0	0.6	99[ab]	91
8	Phe	Trp	Glu	Arg		
	—	—	—	—	100[a] (174 g)	100 (315 g)
	1.0	—	—	—	90[ab]	90
	—	1.0	—	—	90[ab]	89
	—	—	5.0	—	100[a]	95
	—	—	10.0	—	75[bc]	76
	1.0	—	—	0.6	102[a]	92
	—	1.0	—	0.6	95[ab]	91
	—	—	10.0	0.6	69[c]	72
9	Leu	Glu	Lys	Arg		
	—	—	—	—	100[a] (203 g)	100 (392 g)
	6.0	—	—	—	96[a]	92
	—	10.0	—	—	96[a]	93
	—	—	0.4	—	71[b]	70
	6.0	—	—	0.6	94[ab]	87
	—	10.0	—	0.6	93[ab]	85
	—	3.0	0.4	—	80[ab]	86
	—	6.0	0.4	—	79[ab]	93

[1] Seven- to twenty-one-day experimental assays.
[2] All amino acids, except DL-methionine, fed as a percentage of the diet in the L-form. Levels of basic amino acids refer to mono-hydrochlorides (histidine-monohydrate).
[3] Treatments bearing similar superscripts are not significantly different ($P > 0.05$).
[4] Values in parentheses represent absolute grams gain and grams feed intake per chick.

acids were then made at the expense of glucose to complete the test diets, and with the exception of DL-methionine, only the L- form of amino acid was used.

Procedures were similar in all experiments. Male chicks of the broiler meat type were reared under standard conditions until 7 days of age. At this time, they were fasted overnight, individually weighed and allotted to pens at random from within weight groups. Three replicates of 10 chicks were assigned to each treatment and diets were offered ad libitum for the period 7 to 21 days. Gain data were analyzed by analysis of variance and differences between treatment means subjected to Duncan's multiple range test (13).

RESULTS AND DISCUSSION

Preliminary experiments to establish the level of each amino acid which would create a condition of "imbalance" have been omitted for the sake of brevity. Significant growth depressions were ultimately obtained with excess levels of lysine, tyrosine, histidine, glycine, methionine, cystine, and threonine. Less marked growth depressions were observed with excess levels of valine, isoleucine, phenylalanine, tryptophan and leucine (table 2). Glutamic acid at 10% of the diet brought about a significant growth depression in experiment 8 but not in experiment 9. No explanation for this discrepancy is available at this time. Amounts of these amino acids required to bring about indications of an imbalance ranged from as little as 0.4% of the diet (L-lysine·HCl) to as much as 6.0% (L-leucine), or even 10% (L-glutamic acid), with most in the range of 1.0 to 2.0%. Three classes of imbalance apparently occurred: (a) those which produced a greater percentage growth reduction than percentage feed intake reduction (lysine, tyrosine, histidine, methionine, cystine, and possibly valine); (b) those which brought about a propor-

TABLE 3

The role of arginine in the detoxification mechanism [1]

Exp. no.	Amino acid added to basal diet [2]				Gain [3]	Feed consumption
	%	%	%	%	*% of basal*	*% of basal*
10	Glu	Gly	Leu	Arg		
	1.0	—	—	—	100[a] (190 g) [4]	100 (351 g)
	1.0	1.0	—	—	103[a]	94
	1.0	—	1.0	—	103[a]	97
	1.0	1.0	1.0	—	101[a]	94
	1.0	—	—	0.5	111[a]	97
	1.0	1.0	—	0.5	105[a]	93
	1.0	—	1.0	0.5	104[a]	96
	1.0	1.0	1.0	0.5	104[a]	94
11	His	Arg	Met			
	—	—	—		100[a] (201 g)	100 (370 g)
	1.6	—	—		66[c]	79
	1.6	0.9	—		89[ab]	82
	1.6	1.2	—		84[b]	80
	1.6	0.9	0.10		84[b]	80
	1.6	0.9	0.15		81[b]	79
	1.6	1.2	0.10		84[b]	78
	1.6	1.2	0.15		85[b]	81
12 [5]	Tyr	Arg	Met			
	—	—	—		100[a] (209 g)	100 (381 g)
	3.0	—	—		69[c]	82
	3.0	0.9	—		80[b]	86
	3.0	1.2	—		85[b]	87
	3.0	1.5	—		84[b]	85
	3.0	0.9	0.2		83[b]	86
	3.0	1.2	0.2		81[b]	85
	3.0	1.5	0.2		78[bc]	83

TABLE 3 (*Continued*)

The role of arginine in the detoxification mechanism [1]

Exp. no.	Amino acid added to basal diet [2]			Gain [3]	Feed consumption
	%	%	%	*% of basal*	*% of basal*
13	Arg				
	—			100[a] (203 g)	100 (379 g)
	0.5			106[a]	97
	1.0			103[a]	96
	1.5			100[a]	93
	2.0			97[a]	90
	2.5			99[a]	94
	3.0			100[a]	93
	3.5			93[a]	87
14	Arg	Lys			
	—	—		100[a] (158 g)	100 (332 g)
	3.5	—		99[a]	93
	5.0	—		91[ab]	87
	5.0	0.4		87[bc]	84
	5.0	0.8		78[cd]	79
	5.0	1.2		75[d]	77
	5.0	1.6		77[cd]	79
	5.0	2.0		74[d]	77
15	Lys	His			
	—	—		100[a] (193 g)	100 (347 g)
	0.4	—		82[b]	90
	0.4	0.1		71[bcd]	82
	0.4	0.3		76[bc]	85
	0.4	0.5		66[cd]	79
	0.4	0.7		59[d]	76
	0.4	0.9		56[d]	76
	0.4	1.1		68[bcd]	78
16	Lys	His	Arg		
	—	—	—	100[a] (202 g)	100 (353 g)
	0.4	—	—	65[b]	76
	0.4	1.2	—	53[b]	64
	0.4	1.2	0.30	85[a]	82
	0.4	1.2	0.45	89[a]	87
	0.4	1.2	0.60	91[a]	86
	0.4	1.2	0.75	92[a]	86
	0.4	1.2	0.90	92[a]	87
17	Lys	Orn			
	—	—		100[a] (156 g)	100 (288 g)
	0.4	—		67[b]	81
	0.4	0.1		69[b]	81
	0.4	0.2		65[bc]	79
	0.4	0.3		49[c]	68
	0.4	0.4		55[bc]	74
	0.4	0.5		54[bc]	70
	0.4	0.6		53[bc]	68

[1] Seven- to twenty-one-day experimental assays.
[2] All amino acids, except DL-methionine, fed as a percentage of the diet in the L-form. Levels of basic amino acids refer to mono-hydrochlorides (histidine-monohydrate).
[3] Treatments bearing similar superscripts are not significantly different ($P > 0.05$).
[4] Values in parentheses represent absolute grams gain and grams feed intake per chick.
[5] Seven- to twenty-day assay.

tional decrease in feed intake and weight gain (glycine, threonine, isoleucine, phenylalanine, and tryptophan); and (c) those which produced a greater feed intake depression than weight gain depression (possibly leucine and glutamic acid).

Superficially, at least, there appeared to be no common denominator underlying the degree of toxicity among amino acids, i.e., molar equivalents of various amino acids or of their nitrogenous groups did not result in equivalent toxicities, nor was there any relationship when toxic levels were expressed as a percentage of the chicks' requirement. The relatively high levels of leucine fed without effecting an imbalance and the lack of response to arginine under these conditions could be a manifestation of a particular role for leucine, the knowledge of which might explain its apparent antagonism with isoleucine and valine, as observed by Harper et al. (1).

The aforementioned conditions of imbalance, excluding those of tryptophan, leucine and glutamic acid, were all corrected to some degree by the addition of arginine. The level of arginine required to bring about this improvement ranged from 0.3% to 2.0% but did not appear to be dependent upon the severity of the imbalance. In imbalances defined previously as class (a), the first level of extra arginine had a greater effect on growth than on feed consumption. This resulted in a restoration of the proportional relationship between gain and feed intake.

The very fact that some conditions of imbalance required as high as 0.9% arginine to maximize growth response indicates that the role of arginine is not one of simply an arginine deficiency for protein synthesis. Furthermore, if the cutback in feed intake on the imbalanced rations did decrease arginine intake to below required levels it is likely that secondary and tertiary deficiencies would quickly become obvious. To explore this possibility experiment 10 was conducted to assure that arginine and certain other amino acids, i.e., those not demonstrating imbalance tendencies readily, were not limiting in the original basal diet. Since there was no response to these amino acids (table 3), it was felt that only arginine and methionine had the potential of becoming limiting if feed intake was reduced. Experiments 11 and 12 were then conducted and illustrated that methionine did not become limiting under the reduced intake of an imbalanced diet even after the arginine response had been effected. Thus the role of arginine cannot be explained as one of a simple reduction in arginine intake.

The hypothesis that arginine mediates its effects by transport inhibition at either the absorption or at the reabsorption site was studied in experiments 13, 14, and 15. In experiment 13, a level of at least 3.5% arginine was required to demonstrate a possible growth depression due to excess arginine. If arginine competes with lysine at either the absorption or reabsorption sites the mechanism should operate in reverse. To test the hypothesis the level of excess arginine was raised to 5% and then graded levels of lysine were added. If there was, in fact, an arginine-induced imbalance present, lysine did not relieve this imbalance condition. In fact there was a reduction in growth with graded levels of additional lysine. According to the classical concept of mechanisms for the transport of dibasic, dicarboxylic, neutral and imino and glycine amino acids (14), arginine should only compete with the dibasic amino acids. In actual fact, however, it counteracted the toxic effects of all four groups of amino acids. Furthermore, histidine, another basic amino acid, was not effective in counteracting a lysine toxicity (exp. 15). It is evident from the above that the competitive theory for the role of arginine is not supported by the data.

If the chick possesses a latent urea cycle it is possible that the imbalance condition might activate such a cycle and an increased need for urea cycle intermediates would result. To test this final hypothesis, ornithine, an intermediate in the urea cycle, was added to a lysine imbalanced diet (exp. 17). No benefit was secured from the additional ornithine and in fact higher levels of ornithine appeared detrimental. Thus the suggestion that arginine plays a role in detoxification or excretion of excess amino acids via increased urea cycle activity (5) is not necessarily supported. In addition, it is extremely difficult to understand from examination

of the precursors of uric acid, the major protein degradation route in the chicken, why arginine should be more stimulating than any other amino acid for this cycle.

The only comment possible in the light of these findings is to suggest that an alternative disposal mechanism must be available to the chick which utilizes arginine or its metabolic by-products. This mechanism apparently can be saturated by excess amino acids and when this point is reached further additions of arginine are of no value. It is hoped that further investigation will elucidate the role of arginine in amino acid imbalance states.

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Vitamin B_6 Requirement of the Mink [1,2]

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ABSTRACT The requirement and metabolism of vitamin B_6 were investigated with growing mink. Purified diets containing zero, 0.75, 1.5, 3.0 and 6.0 mg/kg of vitamin B_6 (pyridoxine·HCl) were fed to 50 pastel mink kits. Mink receiving no vitamin B_6 showed deficiency symptoms (ataxia, acrodynia, convulsions, irritability and apathy) terminating in death unless relieved by supplementary vitamin B_6. Groups receiving diets containing 0.75 mg/kg of vitamin B_6 showed growth comparable to those on higher levels but developed some abnormal symptoms. In a further study 75 pastel male mink kits were fed purified diets containing zero, 0.4, 0.8, 1.6, and 3.2 mg/kg of vitamin B_6. Mink receiving the zero, 0.4 and 0.8 levels of vitamin B_6 showed deficiency symptoms. The group receiving 0.8 mg/kg showed growth comparable to the higher groups; however, only 9 animals out of 15 survived at 20 weeks of age. Forty male mink were used in two metabolism studies in which urinary xanthurenic acid, kynurenic acid and N^1-methylnicotinamide were measured. Urinary excretion of xanthurenic and kynurenic acids was sharply elevated following the ingestion of 2.5 mmoles L-tryptophan during vitamin B_6 depletion. Supplementation with 0.8 mg/kg of vitamin B_6 brought about a slight reduction in the urinary excretion of xanthurenic and kynurenic acids while the addition of 1.6 and 3.2 mg/kg brought about an immediate return to predepletion levels. Urinary excretion of N^1-methylnicotinamide was slightly increased following the ingestion of tryptophan in all dietary treatments. The lowest dietary level of vitamin B_6 which promoted growth and prevented abnormal symptoms and abnormal tryptophan metabolism was 1.6 mg/kg.

Little information is available regarding the vitamin B_6 requirement of the mink (*Mustela vison*). The National Research Council's recommended level (1) is based on the work of Tove et al. (2) which indicated a diet containing 2 mg of pyridoxine per kilogram was satisfactory for growing mink kits.

The present experiments with mink were designed (a) to obtain more information on the vitamin B_6 requirement, (b) to describe the effect of vitamin B_6 deficiency, and (c) to study tryptophan metabolism during B_6 deficiency.

EXPERIMENTAL

Three experiments were conducted. The first two were growth trials in which the criteria included feed consumption, weight gains, deficiency symptoms, and pathological lesions. Five levels of vitamin B_6 were used, with the levels in experiment 1 approximately double those in succeeding experiments. Experiment 3 was a metabolism study in which the urinary excretion of kynurenic acid, xanthurenic acid and N^1-methylnicotinamide was measured.

Experiment 1. Twenty-five male and 25 female pastel mink kits were randomly assigned to 5 experimental treatments balanced as to weight and sex. The purified diets (table 1) contained zero, 0.75, 1.5, 3.0 and 6.0 mg of vitamin B_6 as pyridoxine hydrochloride per kilogram of diet and were designated B_6 0, B_6 0.75, B_6 1.5, B_6 3.0 and B_6 6.0, respectively. Mink were introduced to the purified diet by adding increasing amounts of it to the regular ranch diet over a 2-week transition period. By 10 weeks of age they were receiving only the purified diet. The animals were housed in wire cages in a covered shed. Feed was offered in earthenware crocks every other day. Individual feed consumption and weekly weight records were kept and ani-

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TABLE 1

Composition of purified diet

Ingredients	%
Vitamin-free casein [1]	30.00
Dextrose [2]	39.00
L-Arginine [1]	0.50
DL-Methionine [1]	0.25
L-Cystine [1]	0.25
Powdered cellulose [3]	5.00
Lard [4]	10.00
Cottonseed oil [5]	9.00
Minerals (Phillips and Hart, salts IV) [1]	5.00
Vitamin mixture [6]	1.00

[1] General Biochemicals, Inc., Chagrin Falls, Ohio (27).
[2] Cerelose, Corn Products Company, New York.
[3] Solka Floc, Brown Company, Berlin, New Hampshire.
[4] Tobin Packing Company, Rochester, New York.
[5] Wesson Oil, Hunt-Wesson Foods, Fullerton, California.
[6] To provide per kg of diet: thiamine·HCl, 10 mg; pyridoxine·HCl, 0–6.0, 0.75, 1.5, 3.0 or 6.0 mg; riboflavin, 20 mg; Ca D-pantothenate, 15 mg; niacin, 40 mg; *i*-inositol, 250 mg; *p*-aminobenzoic acid, 500 mg; menadione, 25 mg; folic acid, 2 mg; vitamin B_{12}, 40 μg; biotin, 500 μg; vitmain E, 40 IU; vitamin D_3, 1200 IU; vitamin A palmitate, 12000 IU; L-ascorbic acid, 99 mg; choline di-hydrogen citrate, 6.6 g; and Santoquin, 187.5 mg.

mals that died during the experiment were autopsied as soon as possible after death.

The study was continued for 15 weeks. After 11 weeks, 3 of the males and 4 of the females originally assigned to the B_6 0 diet had died, and the survivors were changed to the B_6 6.0 diet.

Experiment 2. Seventy-five male pastel mink kits were randomly assigned to 5 experimental treatments from weight groups. The purified diets in this experiment contained zero, 0.4, 0.8, 1.6 and 3.2 mg of vitamin B_6 as pyridoxine hydrochloride per kilogram of diet and were designated B_6 0, B_6 0.4, B_6 0.8, B_6 1.6 and B_6 3.2, respectively. Feed consumption, body weight and autopsy records were kept in a manner similar to experiment 1. A 2-week transition period was used to transfer the mink from the ranch diet to the purified diet and the mink were receiving only the purified diet at 8 weeks of age. Then, to reduce body storage of vitamin B_6, the mink were fed the B_6 0 diet for 2 weeks and were introduced to their designated diets at 10 weeks of age.

The experiment was terminated for the group receiving the B_6 0 diet after 2 weeks (4 weeks on B_6 0 diet) and after 6 weeks (8 weeks on experiment) for the B_6 0.4 groups due to high mortality and the occurrence of severe deficiency symptoms. The remaining groups were removed from the experiment after 10 weeks.

Experiment 3. Two metabolism trials were conducted, in each of which 20 male mink were randomly assigned to the 5 diets used in experiment 2. Each trial was of 5 weeks' duration. Trial 1 consisted of a 1-week preliminary period in which all the animals received the B_6 3.2 diet, a 1-week depletion period in which all the animals received the B_6 0 diet, and a 3-week period in which the animals received the previously assigned diet. Trial 2 consisted of a 1-week preliminary period, a depletion period lengthened to 2 weeks and a 2-week period in which the animals received the previously assigned diet.

Two consecutive 24-hour urine samples were collected from each mink at the end of each week. Following the first 24-hour collection a loading dose of L-tryptophan was administered before the second 24-hour urine sample was obtained. L-Tryptophan was mixed with the B_6 0 diet at the rate of 2% and this diet was offered to each animal in an amount to provide about 2.5 mmoles L-tryptophan/kg body weight.

Urine was collected under toluene in amber polyethylene bottles and stored at −30°. The urinary excretion of xanthurenic acid preceding and following the tryptophan load was measured each week using the procedure of Wachstein and Gudaitis (3). The Wachstein and Gudaitis method provides a rapid colorimetric measurement for urinary xanthurenic acid, but is not considered to be as precise as the more recent fluorometric method of Satoh and Price (4). The latter method, however, is extremely tedious and time-consuming, but it does provide a measurement of kynurenic acid as well as xanthurenic acid. The Wachstein and Gudaitis method therefore was used to provide a rapid assay of xanthurenic acid excretion and this was later verified by using the Satoh and Price method. Because of the time-consuming feature of the latter method, a decision was made not to analyze all the samples but rather to analyze only the samples from each group after the dietary treatment had been imposed for 2

weeks following depletion, and from the B_6 3.2 group throughout the complete cycle of depletion and repletion. This provided a representative picture of the changes which occurred in urinary xanthurenic and kynurenic acid levels during depletion and repletion and demonstrated that these changes were reversible. The excretion of N^1-methylnicotinamide was also measured after 2 weeks of supplementation following depletion using the method of Vivian et al. (5).

Analyses of variance were carried out on the data and significant differences were calculated according to the procedure described by Duncan (6).

RESULTS AND DISCUSSION

Experiment 1. Deficiency symptoms appeared in the B_6 0 group in about 2 weeks. These signs included reduced feed intake, reduced weight gain, diarrhea, brown exudate around the nose, excessive lacrimation and difficulty in opening the eyes, swelling and puffiness around the nose and facial region, apathy, muscular incoordination, convulsions and finally death unless relieved by supplementary vitamin B_6. On postmortem examination a number of nonspecific pathological changes were observed including fatty livers, enlarged spleen, congested lungs and pale kidneys. Microscopically, the liver showed fatty degeneration with pyknotic nuclei scattered throughout, the spleen showed hemosiderosis and myeloid metaplasia and the kidney showed fatty infiltration of tubule cells.

Severe symptoms such as those described above were not observed in the other dietary groups. At least one male mink in the B_6 0.75 group, however, became extremely irritable after 10 weeks on the diet. Another male in the B_6 0.75 group exhibited a severe convulsion at 15 weeks.

Growth and feed consumption data are summarized in table 2. Both the males and females in the B_6 0 group gained less weight ($P < 0.05$) and consumed less feed at week 5 than the other groups. There were no significant differences in feed consumption or weight gains among the other groups. The mean weights of the males in the B_6 0, B_6 0.75, and B_6 1.5 groups are shown in figure 2. The data from the B_6 3.0 and B_6 6.0 males were similar to those in the B_6 1.5 group and were omitted from figure 1 for simplicity. The 2 males and 1 female remaining in the B_6 0 group after 11 weeks on experiment were transferred to the B_6 6.0 diet. They exhibited a rapid and dramatic remission of symptoms and growth response so that after 15 weeks on experiment their body weights were comparable to those in the other groups (fig. 1 for the males).

The animals in experiment 1 had not been subjected to a vitamin B_6-depletion period. This would suggest that the symptoms which were observed in 2 of the males in the B_6 0.75 groups may have

TABLE 2

Summary of growth and feed data of mink receiving purified diets containing different levels of vitamin B_6 (exp. 1)

Vitamin B_6 content of diet	Sex	Avg net gain			Avg feed consumed		
		5 weeks	10 weeks	15 weeks	5 weeks	10 weeks	15 weeks
mg/kg		*g*	*g*	*g*	*g*	*g*	*g*
0	M	182.2 [a] [1] (5) [2]			2520 [a]		
	F	50.8 [c] (5)			1570 [c]		
0.75	M	386.8 [b] (5)	620.2 [a] (5)	690.4 [a] (5)	3083 [ab]	6366 [a]	9224 [a]
	F	350.0 [d] (5)	520.2 [c] (5)	619.2 [c] (5)	2222 [cd]	4595 [c]	6934 [c]
1.5	M	543.8 [b] (5)	827.4 [a] (5)	957.2 [a] (4)	3509 [b]	7434 [a]	11094 [a]
	F	268.0 [d] (5)	358.4 [c] (5)	522.6 [c] (5)	2446 [d]	5026 [c]	7594 [c]
3.0	M	478.5 [b] (4)	745.5 [a] (4)	840.2 [a] (4)	3192 [ab]	6714 [a]	10137 [a]
	F	311.8 [d] (5)	451.8 [c] (5)	459.8 [c] (5)	2645 [d]	5421 [c]	8068 [c]
6.0	M	534.6 [b] (5)	860.4 [a] (5)	972.2 [a] (5)	3314 [ab]	6994 [a]	10495 [a]
	F	304.4 [d] (5)	444.0 [c] (5)	563.4 [c] (5)	2442 [d]	5016 [c]	7701 [c]

[1] Numbers in the same column with a common superscript are not statistically different ($P < 0.05$).
[2] Parentheses indicate the numbers of animals included in this average.

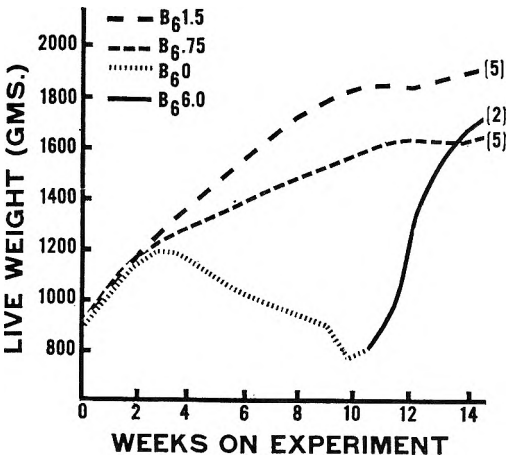

Fig. 1 Mean weights of male mink receiving purified diets containing different levels of vitamin B_6 (exp. 1). Figures in parentheses indicate numbers of animals weighed at the times indicated.

been delayed as a result of vitamin B_6 storage as described in the rat by Cerecedo and Foy (7) and by Morrison and Sarett (8). Because of this and the somewhat erratic growth of males at this level, it seemed reasonable to conclude that 0.75 mg of vitamin B_6 per kilogram of diet was marginal and that the levels of 1.5, 3.0 and 6.0 mg/kg were adequate or in excess of that required for normal growth and health. It also suggested that in future studies an experimental period with graded levels of vitamin B_6 should be preceded by a depletion period.

Experiment 2. The onset of deficiency symptoms in experiment 2 was quite rapid and they were primarily of the nervous type such as ataxia, convulsions and coma rather than the severe facial symptoms observed in experiment 1. The difference in syndromes was attributed to the younger age at which the animals were introduced to the purified diet, or to the depletion period which preceded the actual dietary treatments, or both. The postmortem lesions were similar to those observed in experiment 1. The mean weights of the dietary groups are shown in figure 2. At 3 weeks, 7 of the original 15 males in the B_6 0.4 group had died and the remainder were removed from the experiment. A summary of weight gains and feed consumption at weeks 4, 6 and 10 is shown in table 3. The B_6 0.4 group gained less weight ($P < 0.05$) and consumed less feed ($P < 0.05$) than the other groups at week

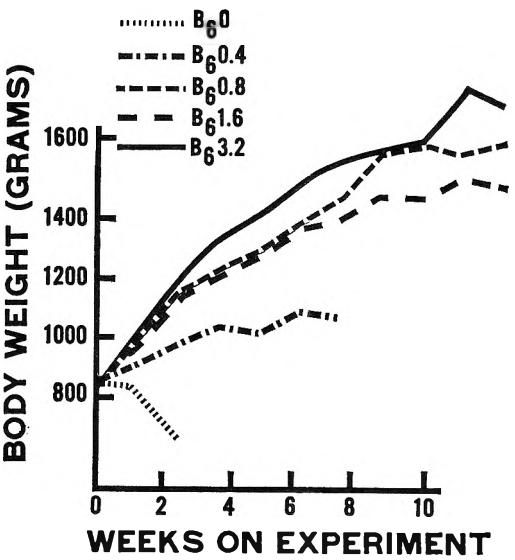

Fig. 2 Mean weights of male mink receiving purified diets containing different levels of vitamin B_6 (exp. 2).

TABLE 3

Summary of growth and feed data of male mink receiving purified diets containing different levels of vitamin B_6 (exp. 2)

Vitamin B_6 content of diet	Mean net gain			Mean feed consumed		
	4 weeks	6 weeks	10 weeks	4 weeks	6 weeks	10 weeks
mg/kg	*g*	*g*	*g*	*g*	*g*	*g*
0						
0.4	131.8 [a] [1] (12) [2]			1549 [a]		
0.8	407.7 [b] (15)	433.8 [a] (13)	544.6 [a] (9)	1840 [b]	2943 [a]	5349 [a]
1.6	376.4 [b] (14)	440.5 [a] (13)	519.6 [a] (11)	2155 [c]	3178 [a]	5851 [ab]
3.2	431.6 [b] (14)	565.6 [a] (14)	676.6 [a] (13)	2386 [c]	3707 [b]	6754 [b]

[1] Numbers in the same column with a common superscript are not statistically different ($P < 0.05$).
[2] Parentheses indicate the numbers of animals inclu ded in this mean.

4. The B_6 0.8 group did not differ in weight gains from the other groups at weeks 4, 6 and 10. They did, however, consume less feed at week 4 than the other groups ($P < 0.05$) and less than the B_6 3.2 group at weeks 6 and 10 ($P < 0.05$). When the experiment was terminated at week 10 the 9 survivors of this group (B_6 0.8) showed symptoms similar to those observed in the B_6 0 and B_6 0.4 groups. On this basis it was believed that the B_6 0.8 group, like the B_6 0.75 group in experiment 1, was receiving only marginal amounts of the vitamin.

Experiment 3. When vitamin B_6-deficient mink were administered a loading dose of tryptophan they exhibited a sharp elevation in the excretion of xanthurenic acid. Table 4 indicates the average excretion of xanthurenic acid, as measured by the procedure of Wachstein and Gudaitis (3) in trials 1 and 2, respectively. In trial 1, all groups except the B_6 0.8 group showed this sharp elevation after 1 week of depletion. The first week of depletion did not produce the expected elevation in xanthurenic acid excretion in the B_6 0.8 group. This could be explained only by a storage effect, since all groups had received identical dietary treatment up to this time. This group was, therefore, held on the deficient diet for a second week which prompted a marked rise in urinary xanthurenic acid. In the B_6 0 group the initial elevation increased somewhat and leveled off between weeks 3 and 5. Repletion with 0.4 mg/kg did not reduce the excretion of xanthurenic acid at all. The mink in both groups showed no gross symptoms of the vitamin deficiency and their appetites were not impaired during this short period. Following supplementation, the B_6 0.8 group excreted smaller amounts of xanthurenic acid ($P < 0.05$) but this excretion did not return to predepletion levels. Excretion in the B_6 1.6 and B_6 3.2 groups returned to predepletion levels within 1 week after supplementation.

In trial 2, all groups were fed the depletion diet (B_6 0) for 2 weeks following the preliminary week. All groups excreted larger amounts ($P < 0.05$) of xanthurenic acid at the end of the second depletion week. The B_6 0 and B_6 0.4 groups then lev-

TABLE 4

Mean 24-hour urinary excretion of xanthurenic acid following a tryptophan load (method of Wachstein and Gudaitis, 1952)

Dietary vitamin B_6 level during repletion	Week number 1	2	3	4	5
	Stage of nutrition				
	Preliminary (B_6 3.2)	Depletion (B_6 0)	Repletion (indicated diets)		
mg/kg diet	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*
			Experiment 3, trial 1		
0	3.0 [a 1]	134.1 [b]	335.4 [c]	388.0 [c]	346.4 [c]
0.4	2.8 [a]	214.6 [bc]	234.9 [bc]	291.6 [c]	201.7 [b]
0.8	3.8 [a]	8.1 [a]	(260.9 [c]) [2]	184.2 [b]	109.6 [b]
1.6	3.0 [a]	105.1 [b]	17.8 [a]	1.5 [a]	2.2 [a]
3.2	3.6 [a]	145.6 [b]	0 [a]	0.4 [a]	0.9 [a]
			Experiment 3, trial 2		
	Preliminary	Depletion		Repletion	
0	0.3 [a 1]	84.6 [ab]	197.4 [cd]	161.0 [bc]	262.0 [d]
0.4	0 [a]	109.6 [b]	194.1 [bc]	155.1 [bc]	212.9 [c]
0.8	0 [a]	38.3 [a]	158.3 [b]	80.1 [ab]	48.1 [a]
1.6	0 [a]	75.4 [ab]	155.2 [b]	1.5 [a]	0 [a]
3.2	0 [a]	78.2 [ab]	126.4 [b]	0.2 [a]	0 [a]

[1] Means in the same row or column with a common superscript are not statistically different ($P < 0.05$).

[2] This value was obtained following a second week of depletion and the group was thus repleted for only 2 weeks (see text).

eled off or increased slightly during the following 2 weeks. Once again, no gross symptoms of vitamin deficiency were observed and feed consumption was comparable with other groups. The xanthurenic acid excretion of the B_6 0.8 group decreased slowly following supplementation, whereas that of the B_6 1.6 and B_6 3.2 groups returned to predepletion levels immediately following supplementation.

The mean 24-hour excretion of kynurenic acid and xanthurenic acid, as measured by the procedure of Satoh and Price (4), preceding and following tryptophan loading for trials 1 and 2 after vitamin B_6 depletion and 2 weeks of supplementation, is shown in table 5. The urinary excretion of both kynurenic acid and xanthurenic acid was elevated following the tryptophan load ($P < 0.05$) in the B_6 0, B_6 0.4 and B_6 0.8 groups but not in the B_6 1.6 or B_6 3.2 groups. The mean excretion of both metabolites, for all dietary groups together was higher ($P < 0.05$) following the ingestion of the tryptophan. The mean excretion of 29.5 mmoles of xanthurenic acid by the B_6 0 group before the tryptophan load suggests that, at that point, this group was not capable of metabolizing the tryptophan contained in the regular diet.

The pattern of urinary xanthurenic and kynurenic acid excretion for the B_6 3.2 group in trials 1 and 2 is shown in table 6. The excretion of both metabolites was elevated ($P < 0.05$) following the ingestion of the tryptophan after 1 week on the vitamin B_6-deficient diet, but returned to levels not significantly greater than preliminary levels after supplementation.

Table 7 shows the mean 24-hour excretion of N^1-methylnicotinamide preceding and following the ingestion of tryptophan in trials 1 and 2. These results suggest that while the mink may be capable of converting trace amounts of tryptophan to niacin, the rat is 150 times more efficient (9). Since it has been shown that mink require a dietary source of niacin (10), the conversion of dietary tryptophan to niacin is clearly inadequate to meet the niacin requirement of this species.

GENERAL DISCUSSION

The absence of dermatitis in both growth experiments, together with the fact that the diets contained 10% of lard and 9% of cottonseed oil, is in agreement

TABLE 5

Mean 24-hour urinary excretion of kynurenic and xanthurenic acids, preceding and following a tryptophan load [1] (method of Satoh and Price, 1958)

Dietary vitamin B_6 level during repletion	Kynurenic acid		Xanthurenic acid	
	Before tryptophan	After tryptophan	Before tryptophan	After tryptophan
mg/kg diet	*μmoles*	*μmoles*	*μmoles*	*μmoles*
		Experiment 3, trial 1		
0	2.5 $^{a\,2}$	57.3 ^{c}	29.5 $^{a\,2}$	421.6 ^{b}
0.4	3.2 ^{a}	105.0	4.6 ^{a}	325.0 ^{c}
0.8	2.4 ^{a}	38.1 ^{bc}	0.8 ^{a}	138.9 ^{d}
1.6	4.5 ^{a}	22.6 ^{ab}	0.8 ^{a}	6.4 ^{a}
3.2	5.8 ^{a}	22.3 ^{ab}	0.7 ^{a}	6.6 ^{a}
Mean	3.7 $^{m\,3}$	49.0 ^{n}	7.3 ^{m}	179.7 ^{n}
		Experiment 3, trial 2		
0	1.1 $^{a\,2}$	45.0 ^{cd}	22.6 $^{a\,2}$	363.7 ^{b}
0.4	2.0 ^{a}	57.2 ^{d}	1.0 ^{a}	335.3 ^{b}
0.8	2.0 ^{a}	30.1 ^{bc}	0.3 ^{a}	97.6 ^{a}
1.6	4.1 ^{a}	14.6 ^{ab}	0.7 ^{a}	10.7 ^{a}
3.2	3.2 ^{a}	10.5 ^{ab}	0.6 ^{a}	3.1 ^{a}
Mean	2.4 $^{m\,3}$	31.9 ^{n}	3.4 ^{m}	155.8 ^{n}

[1] Mink depleted of vitamin B_6 for 1 to 2 weeks and repleted as indicated for 2 weeks (see table 4 for details).

[2] Means for a given metabolite in the same row or column with a common superscript are not statistically different ($P < 0.05$).

[3] m significantly less than n ($P < 0.05$) for each metabolite.

TABLE 6

Pattern of 24-hour urinary excretion of kynurenic and xanthurenic acids preceding and following a tryptophan load, during vitamin B_6 depletion and repletion

Stage of nutrition	Dietary vitamin B_6 level	Kynurenic acid		Xanthurenic acid	
		Before tryptophan	After tryptophan	Before tryptophan	After tryptophan
	mg/kg	*μmoles*	*μmoles*	*μmoles*	*μmoles*
		Experiment 3, trial 1			
Preliminary	3.2	4.6 [a 1]	13.6 [ab]	0.9 [a 1]	3.2 [a]
Depletion	0	1.1 [a]	41.2 [c]	0.2 [a]	160.3 [b]
Treatment Week 1	3.2	3.9 [a]	15.7 [b]	1.2 [a]	5.1 [a]
Treatment Week 2	3.2	5.8 [a]	22.3 [b]	0.7 [a]	6.6 [a]
Treatment Week 3	3.2	6.4 [a]	24.8 [b]	2.1 [a]	7.2 [a]
Means [2]		4.4 [m]	23.5 [n]	1.0 [m]	36.5 [n]
		Experiment 2, trial 2			
Preliminary	3.2	5.1 [a 1]	16.6 [a]	1.0 [a 1]	3.5 [a]
Depletion Week 1	0	2.2 [a]	54.5 [b]	0.5 [a]	72.3 [a]
Depletion Week 2	0	1.2 [a]	66.8 [b]	0.8 [a]	219.2 [b]
Treatment Week 1	3.2	2.6 [a]	12.0 [a]	0.4 [a]	2.8 [a]
Treatment Week 2	3.2	3.2 [a]	10.5 [a]	0.6 [a]	3.1 [a]
Means [2]		2.8 [m]	32.9 [n]	0.7 [m]	63.2 [n]

[1] Means for the same metabolite in the same row or column with a common superscript are not statistically different ($P < 0.05$).
[2] m significantly ($P < 0.05$) less than n for each metabolite.

TABLE 7

Mean 24-hour excretion of N^1-methylnicotinamide preceding and following a tryptophan load [1] (exp. 3, trials 1 and 2)

Dietary vitamin B_6 level during repletion	Trial 1		Trial 2	
	Before tryptophan	After tryptophan	Before tryptophan	After tryptophan
mg/kg diet	*μmoles*	*μmoles*	*μmoles*	*μmoles*
0	1.08 [a 2]	2.27 [b]	1.10 [ac 2]	1.52 [bc]
0.4	0.59 [a]	1.68 [bc]	0.64 [a]	1.11 [ab]
0.8	1.00 [a]	1.3 [ac]	0.60 [a]	0.66 [a]
1.6	0.86 [a]	1.10 [ac]	0.81 [a]	1.04 [ab]
3.2	0.76 [a]	1.32 [ac]	0.62 [a]	0.82 [a]
Mean [3]	0.86 [m]	1.54 [n]	0.70 [m]	0.96 [n]

[1] Mink depleted of vitamin B_6 for 1 to 2 weeks and repleted for 2 weeks as indicated (see table 4 for details).
[2] Means in the same row or column within each trial with a common superscript are not statistically different ($P < 0.05$).
[3] m is significantly less than n ($P < 0.05$) within each trial.

with the findings of other workers (11–13). These workers suggested that the dermatitis often observed in vitamin B_6 deficiency was related to fatty acid synthesis which was in some manner impaired by vitamin B_6 deficiency. The fatty livers observed in postmortem examinations of vitamin B_6-deficient mink are consistent with the observations of Engel (14) who concluded that pyridoxine as

well as choline and essential fatty acids were necessary to prevent fatty livers in the rat.

The congestion of the lungs observed in the postmortem examination of several mink suggests that vitamin B_6 deficiency may lower the resistance to infection. This has been suggested by previous investigators (15–18). This would indicate that some of the deaths and nonspecific postmortem lesions attributed to vitamin B_6 deficiency could actually be due to general infection to which the animal was rendered more susceptible by the vitamin deficiency.

It has been reported (19–21) that the cat does not excrete kynurenic acid or xanthurenic acid. It has also been reported (22) that the cat does not convert tryptophan to niacin. The inability of the mink to survive without a dietary source of niacin has, therefore, led to the assumption that tryptophan metabolism in the mink is similar to that in the cat. The human (23) shows an elevation of both these metabolites during vitamin B_6 deficiency but can convert tryptophan to niacin in considerable quantities. It has been reported that the vitamin B_6-deficient dog (24) and the vitamin B_6-deficient pig (25) excrete xanthurenic acid but not kynurenic acid in large quantities following a tryptophan load. But these species can convert tryptophan to niacin in amounts sufficient to supply at least a part of the metabolic requirement. The vitamin B_6-deficient mink, however, shows a sharply elevated urinary excretion of both xanthurenic and kynurenic acids following the ingestion of tryptophan and converts only trace amounts, if any, tryptophan to niacin. These observations suggest that the tryptophan metabolism of the mink does not resemble the cat, human, dog or pig. If we accept the hypothesis of Ikeda et al. (26) that the level of picolinic carboxylase regulates the rate of conversion of tryptophan to niacin, it is possible that this enzyme is limiting in the mink. A more complete study of this metabolic pathway in the mink would seem to be in order.

The results of experiment 2, particularly the occurrence of convulsions, ataxia and coma suggest that 0.8 mg of vitamin B_6 per kilogram of diet was not adequate. The fact that the excretion of kynurenic acid and xanthurenic acids when measured in experiment 3 failed to return to predepletion levels, in mink supplemented at 0.8 mg vitamin B_6/kg of diet, confirms this. We might conclude from these experiments, therefore, that the minimum requirement to promote growth and to prevent the abnormal metabolism of tryptophan in the mink is 1.6 mg of vitamin B_6 (as pyridoxine hydrochloride) per kilogram of diet.

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Niacin Requirement of Growing Mink [1]

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ABSTRACT In studies investigating the niacin requirement and metabolism of growing mink over 180 mink kits receiving a purified diet have shown that the mink requires a dietary source of niacin. The requirement lies between 10 and 20 mg/kg of diet. Survival time on a deficient diet increases with age. Supplementation of a niacin-free diet with either 0.16 or 0.32% DL-tryptophan had no effect in alleviating the niacin deficiency. The mink apparently cannot convert sufficient tryptophan to meet its niacin requirement. Symptoms of niacin deficiency are nonspecific and include anorexia, loss of weight, weakness, coma and death. No discoloration of the buccal mucosa was observed.

This study was designed to determine the niacin requirement of growing mink (*Mustela vison*) using the purified diet technique, and to determine if mink are able to utilize tryptophan as a source of niacin. There are no reports in the literature in which this vitamin has been studied in the mink.

MATERIALS AND METHODS

Experiment 1. A pilot study was conducted to determine whether growing mink require niacin, and to determine the effects of niacin supplementation after they had received a diet deficient in niacin. Ten female mink kits averaging 10 weeks of age were fed a purified diet containing niacin at a level of 40 mg/kg. Ten additional mink were fed a diet with no supplemental niacin. A 2-week transition period was used to change from the normal ranch diet to a purified diet. Details of the diets are included in tables 1 and 2. The levels of vitamins other than niacin varied between experiments 1, 2 and 3, 4. The modification was made to incorporate more recent information about the vitamin requirements of the mink and related species. McCarthy [6] showed, however, that these alterations made no difference in the growth of mink kits. After 3 weeks, 3 of the surviving mink which had received the deficient diet were transferred to the diet containing 40 mg/kg to determine the effect of niacin supplementation therapy.

Experiment 2. This study involved 90 male dark mink kits starting at 8 weeks of age. Five treatment groups received either a niacin-deficient diet, or one of four levels of niacin (10, 20, 30, 40 mg/kg). These diets will hereafter be referred to as N0, N10, N20, etc. Twenty kits were assigned at random to each of the four diets containing niacin and 10 males were assigned to the deficient diet (tables 1 and 2). The animals were weighed at 8, 11, 16, and 29 weeks of age, and daily observations were made as to their general welfare. Food was offered ad libitum. The mink were changed from the regular ranch ration to the purified diet using a 1-day transition period.

Experiment 3. An experiment was conducted with 29 suckling mink kits and their dams. When the kits were 3 weeks of age, the mothers were gradually shifted from the regular ranch diet to the purified diet containing adequate niacin (N40). The transition was completed by the time the kits were 6 weeks of age at

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[6] McCarthy, B. Pantothenic acid requirement and the histopathology of pantothenic acid deficiency in the mink. Ph. D. Thesis, 1964. Cornell University, Ithaca, New York.

TABLE 1

Composition of purified diets fed to growing mink

Ingredient	Exp. 1–2	Exp. 3–4
Vitamin-free casein	30.00	30.00
L-Arginine HCl	0.50	0.50
DL-Methionine	0.25	0.25
L-Cystine	—	0.25
DL-Tryptophan [1]	—	—
Sucrose	39.25	—
Cerelose	—	39.00
Lard	10.00	10.00
Cottonseed oil	9.00	9.00
Corn oil	1.00	—
Powdered cellulose [2]	5.00	5.00
Vitamin mixture [3]	1.00	1.00
Mineral mixture [4]	4.00	5.00
Total	100.00	100.00
	ml/kg diet	*g/kg diet*
Choline chloride	2.0 [5]	—
Choline dihydrogen citrate	—	6.6
	mg/kg diet	*mg/kg diet*
Ethoxyquin [6]	125	125

[1] Varied with experimental treatment (see text).
[2] Solka Floc, BW40, Brown Company, Berlin, New Hampshire.
[3] See table 2.
[4] Phillips and Hart (7) obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.
[5] Potency 70% in aqueous solution.
[6] Santoquin, Monsanto Company, Saint Louis, Mo.

which time they were weaned and taken from their mothers. Thirteen kits were continued on a purified (N40) diet and 16 were placed on a diet deficient in niacin (N0). Kits were weighed at 3-day intervals.

Experiment 4. Forty-five pastel male mink kits were started on experiment when they averaged 11 weeks of age. The transition from ranch to purified diet was completed in 7 days. Fifteen mink were assigned at random to each of three diets, niacin deficient (N0), niacin added at 40 mg/kg (N40) and niacin deficient but containing added DL-tryptophan at a level of 1.6 mg/kg of diet (− N + T). After 5 weeks the niacin-supplemented group was further divided into two groups; (a) niacin at 40 mg/kg, and (b) niacin deficient plus tryptophan at a level of 3.2 mg/kg (− N + T (2 ×)).

RESULTS

Experiment 1. Mink on the niacin-deficient diet promptly began to lose weight (fig. 1). The results of supplementation were dramatic as shown by the rapid recovery of the 3 animals transferred to the

TABLE 2

Vitamin mixture used in mink purified diet (table 1)

Vitamin mix	Exp. 1 and 2		Exp. 3 and 4	
	g	*mg/kg diet* [1]	*g*	*mg/kg diet* [1]
Thiamine·HCl	0.2	2.0	1.0	10.0
Pyridoxine·HCl	0.2	2.0	0.32	3.2
Riboflavin	0.4	4.0	2.0	20.0
Ca D-pantothenate	1.5	15.0	1.5	15.0
Niacin [2]	—	—	—	—
i-Inositol	25.0	250.0	25.0	250.0
p-Aminobenzoic acid	50.0	500.0	50.0	500.0
Menadione	0.5	5.0	2.5	25.0
L-Ascorbic acid	—	—	9.9	99.0
Folic acid	0.10	1.0	0.2	2.0
		µg/kg diet		*µg/kg diet*
Vitamin B_{12} [3]	1.333	13.3	4.0	40.0
Biotin	0.025	250	0.05	500.0
		IU/kg diet		*IU/kg diet*
Vitamin A palmitate (1,000,000 IU/g)	1.2	12,000	—	—
(250,000 IU/g)	—	—	4.80	12,000
Vitamin D_3 (1,000 IU/g)	240.0	2,400	—	—
(250,000 IU/g)	—	—	0.48	1,200
Vitamin E (α-tocopheryl acetate)	4.0	40	—	—
dl-α-tocopheryl acetate-275 IU/g	—	—	18.18	50
Sucrose	675.317	—	880.07	—

[1] When mixed in diet at level of 1%.
[2] Varied with experimental treatment.
[3] 0.1% Triturate in mannitol.

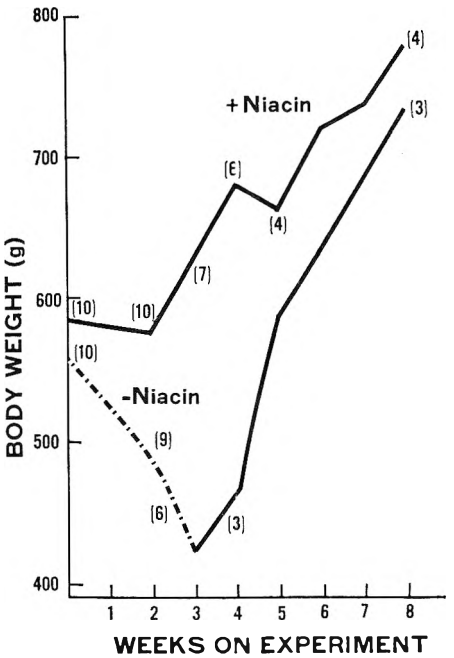

Fig. 1 Body weights of mink fed a diet deficient in niacin (dotted line) or supplemented (40 mg/kg diet) with niacin (solid line). Numbers in parentheses represent numbers of minks weighed at the times indicated. Five mink were sacrificed between weeks 2 and 5 for histological study. One mink died. (Exp. 1.)

niacin-supplemented diet after receiving the deficient diet for 3 weeks.

Experiment 2. Results are shown in table 3. Mink receiving the N0 and N10 diets were all dead by an average of 15 and 24 days on experiment, respectively, Survival was also poorer than expected on the remaining treatments due to the abrupt transition from the meat and fish type diet to the purified diet. Symptoms of deficiency were nonspecific with loss of appetite and loss of weight evident. There was no change of color of the surfaces of the buccal cavity.

Experiment 3. The effects of lack of niacin were clearly evident on the recently weaned kits (fig. 2). Over 50% were dead within 6 days. Symptoms included loss of appetite, loss of weight, weak voice, general weakness and bloody stools. Terminally the animals lost control of their hind legs. Whether this was due to the generalized weakness or was a specific symptom of niacin deficiency could not be determined.

Experiment 4. Both the mink receiving no niacin (N0), and the group receiving

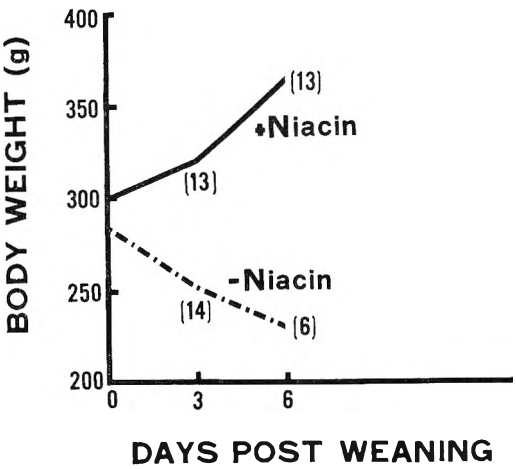

Fig. 2 Body weights of recently weaned mink kits fed a diet deficient in niacin (dotted line) or supplemented (40 mg/kg diet) with niacin (solid line). Numbers in parentheses represent numbers of mink weighed at the times indicated. Eight mink died on the deficient diet between days 3 and 6. (Exp. 3.)

TABLE 3

Weight gain of male mink kits receiving graded levels of niacin (exp. 2)

Diet	No. mink	No. sacrificed	No. died	No. to finish exp.	Avg wt at start	Avg wt 29 wks of age
					g	*g*
N–0 [1]	10	1	9	0 [2]	395	All died
N–10	20	5	15	0 [3]	425	All died
N–20	20	1	7 [4]	12	455	1434
N–30	20	1	3 [4]	16	437	1273
N–40	20	1	6 [4]	13	445	1358

[1] Milligrams of niacin per kilogram diet.
[2] Mean survival time 10 days.
[3] Mean survival time 43 days.
[4] Died early during transition to purified diet (see text).

no niacin but added tryptophan (− N + T), gained weight for about 2 weeks after which they lost weight rapidly (fig. 3). Half of the mink in both groups died in 4 to 5 weeks.

After it became evident that the mink were not able to synthesize adequate niacin from tryptophan at a level of 1.6 g/kg of feed, 13 mink from the N40 groups were divided into two groups. One group continued to receive the N40 diet and the other received the N0 diet to which tryptophan was added at double the previous level of 3.2 g/kg of diet (− N + T (2 ×)). The results shown in figure 4 indicate that the mink were not able to synthesize adequate niacin from tryptophan even when it was fed at this higher level. There were no specific gross deficiency symptoms. The animals developed anorexia, lost weight and gradually grew weaker. Terminally, they became indifferent to their surroundings, went into a coma and died. Figure 5 compares the appearance of an average mink from each of the latter two groups.

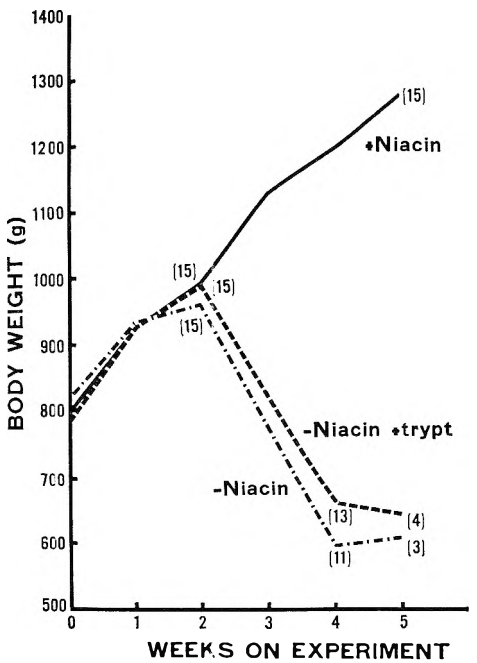

Fig. 3 Body weights of mink fed a diet (a) deficient in niacin, (b) containing adequate niacin (40 mg/kg diet), or (c) containing added DL-tryptophan (1.6 mg/kg diet). Numbers in parentheses represent the numbers of mink weighed at the times indicated. Reduction in the number of experimental animals with time resulted from deaths due to niacin deficiency. (Exp. 4.)

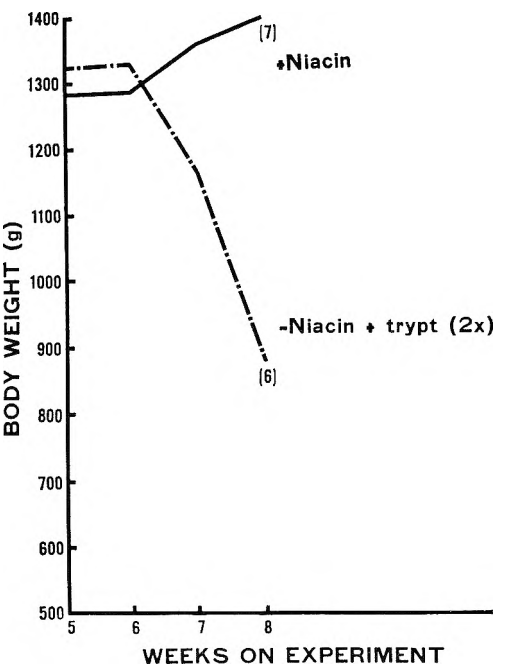

Fig. 4 Body weights of mink which had previously received adequate niacin (40 mg/kg diet) (see fig. 3) but were: (a) changed to a diet deficient in niacin but containing 3.2 g/kg of DL-tryptophan, or (b) maintained on the adequate diet. Numbers in parentheses represent the numbers of mink weighed at the times indicated. (Exp. 4.)

DISCUSSION

The general health of the animals receiving an adequate supplementation of niacin was good with the following exceptions. Due to the unnatural composition and texture of the purified diets there was a certain percentage (about 20%) of the animals in experiment 2 that refused to eat the diet, became emaciated and died soon after they were placed on the experiment. This was due to an attempt to use a 1-day transition period from the ranch to the purified diet. The differences between the treatments in this study, however, were sufficiently large that these early deaths did not materially affect the results of the study. Unless the transition from the ranch diet to the purified diet is

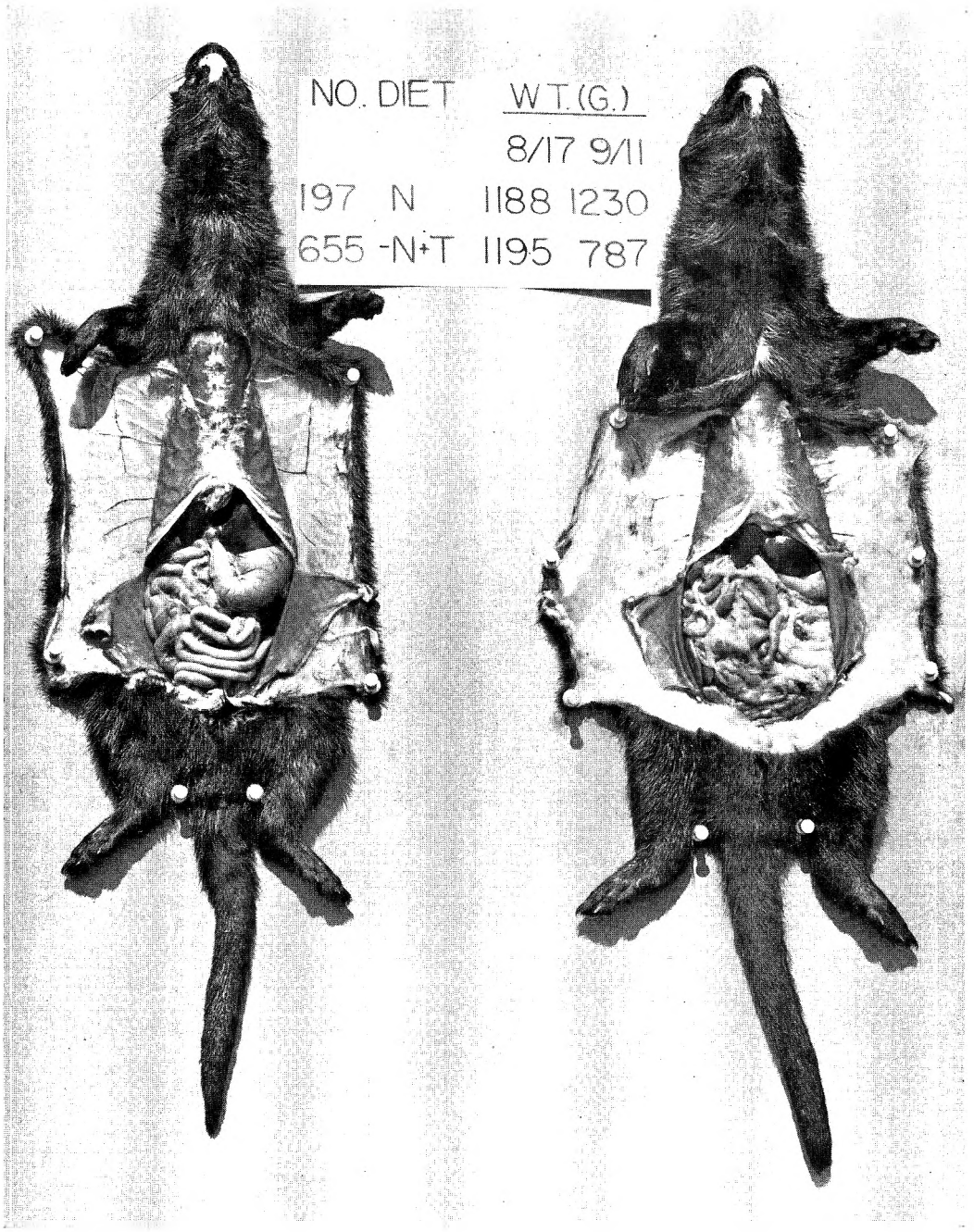

Fig. 5 Representative examples of mink raised on a diet deficient in niacin but containing DL-tryptophan at a level of 3.2 g/kg diet (left) and a diet adequate (40 mg/kg diet) in niacin (right). Pertinent weight data are indicated. Note the thin condition of the deficient animal. (Exp. 4.)

gradual a certain percentage of mink starve rather than adapt themselves to the unnatural diet.

In experiment 4 the time required for half the animals to expire on the deficient diet was between 4 and 5 weeks for the 11-week-old kits. It took only 15 days for the 8-week-old kits to expire in experiment 2. Six-week-old weanling kits expired in less than 6 days on the niacin-deficient diet. From these data it would appear that the survival time increases with age which may or may not be a reflection of level of body stores.

In this study there is little doubt that there was adequate tryptophan available for niacin synthesis. The basal diet (N0) contained 30% casein or about 0.39% tryptophan (1). The added tryptophan (0.16%) was initially calculated to be equivalent to 40 mg of niacin assuming an equivalence of 33 to 40 mg of tryptophan to 1 mg of niacin (2). It is conceivable that the poor performance with tryptophan could have been the result of an imbalanced amino acid pattern. This seems unlikely, however, since with adequate niacin the mink grows well on a diet containing (0.39%) tryptophan.

More recent studies in this laboratory suggest that the mink is capable of converting some tryptophan to niacin. Bowman et al. (3) determined the mean 24-hour urinary excretion of N^1-methylnicotinamide preceding and following the ingestion of 2.5 mmoles of L-tryptophan per kilogram live weight. He found a very slight increase following the tryptophan load. It is clear that the ability to convert tryptophan to niacin is present but it is inadequate to meet the requirement for niacin by this species. The failure to utilize tryptophan as an adequate precursor of niacin places the mink in the same category as the cat (4).

It is of interest that an analysis of mink milk (5) shows a niacin content of 16 mg/100 g of milk which is approximately 20 times that of the cow and twice that of the pig (6).

The essentiality of niacin for mink is clear and the requirement is indicated to be between 10 and 20 mg/kg of diet.

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Protein and Nucleic Acid Metabolism in the Testes of Zinc-deficient Rats [1]

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ABSTRACT The levels and synthesis of protein, RNA, and DNA have been studied in the testes of weanling rats fed zinc-deficient diets during the 2 weeks immediately before attainment of sexual maturity. Control rats were fed zinc-sufficient diets ad libitum or were pair-fed to the deficient rats. Levels of testicular protein, DNA, and RNA were studied at 7 and 14 days after initiation of the zinc-free diets. On day 14 of the experiment, the incorporation of ^{14}C-leucine into testicular protein and ^{14}C-adenine into DNA and RNA was also studied. Histological differences were evident in the testes of the zinc-deficient rats on day 14. The zinc content of the testes of rats fed the zinc-deficient diet was always lower than that of the control groups. No gross impairment of DNA synthesis in the testes was noted early in zinc deficiency. The total protein and RNA content of the testes was reduced in zinc-deficient rats but the incorporation of ^{14}C-leucine and ^{14}C-adenine into protein and RNA, respectively, was unaltered. These data suggest that an increase in protein and RNA catabolism occurs in the zinc-deficient testes rather than a decrease in synthesis.

The consequences of dietary zinc deficiency in the albino rat have been well-delineated in the literature. These include growth retardation, reduced tissue levels of zinc, testicular atrophy, esophageal parakeratosis and a reduction in the activities of various enzymes (1–3). In general, firm biochemical reasons for these changes have not been established.

Recently several investigators have attempted to relate zinc to nucleic acid metabolism and protein synthesis. Thus, reduced synthesis of RNA or protein has been noted in various microorganisms when grown on a zinc-low medium (4–8). Animal studies attempting to show this relation, however, are equivocal (9, 10) and a re-study of this area seemed desirable.

It occurred to us that studies of protein and nucleic acid metabolism in zinc deficiency might be carried out most profitably in the testes of the rat. This organ is a particularly sensitive target in zinc deficiency. Its zinc content is significantly reduced, the seminiferous tubules are atrophied, and a marked reduction in the number of spermatozoa occurs (1, 2). Moreover, Davis and Shami [4] have reported that microsomes in the testes show the greatest depression in zinc content during deficiency. Because these changes occur relatively early in the deficiency, the problem of inanition assumes less importance in the interpretation of results.

The experimental approach was built on the premise that the biochemical effects of zinc deficiency in the testes might be most apparent in the final stages of sperm formation. The minimum dietary zinc requirement of the rat is about 2 ppm (11) and the testes of the rat matures at approximately 45 days of age (12). Accordingly, male weanling rats were fed a diet containing a low amount of zinc but sufficient to permit a normal rate of testicular development until two weeks before day 45 of life. At this point they were divided into groups fed diets of various ade-

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[2] Taken in part from a thesis submitted in partial fulfillment of the requirements for a Ph.D. at Vanderbilt University.

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[4] Davis, J. T., and A. S. Shami 1966 Zinc deficiency and subcellular zinc content in testes. In: Proceedings of Second Symposium on Human Nutrition and Health in the Near East, Beirut, Lebanon (mimeographed).

quacies with respect to zinc and subjected to radioisotope studies designed to evaluate the levels and synthesis of DNA, RNA, and protein.

MATERIALS AND METHODS

Male weanling rats of the Sprague-Dawley strain (21 days of age) were housed individually in all-plastic cages in a temperature- and humidity-controlled room (24° and 50% relative humidity) and fed variations of the basic diet (table 1). This diet assayed approximately 0.5 ppm zinc and is here referred to as the "zinc-deficient" diet. A "low zinc" diet was also used. This diet was prepared by using 10 parts of specially treated casein (13) (zinc content 2.7 ppm) plus 10 parts of commercial casein [5] (zinc content 25.0 ppm). Deionized water was offered ad libitum from plastic water bottles. Zinc was determined on aliquots of organ homogenates by atomic absorption spectroscopy. The organ homogenates were prepared for analysis by a wet-ashing procedure.[6] RNA and DNA were fractionated by the Schmidt-Thannhauser procedure (14) incorporating the modifications suggested by Hutchison et al. (15). The RNA analyses were carried out using the orcinol procedure of Kerr and Seraidarian (16). DNA was measured by the diphenylamine procedure of Schneider (17). Protein was determined by a biuret method (18) using an albumin solution of known nitrogen content as the standard. Acid soluble amino acids were measured by the photometric ninhydrin procedure of Moore and Stein (19). In the isotopic incorporation studies, the proteins were precipitated from testes homogenates by adding an equal volume of 10% trichloracetic acid solution, followed by treatment of the precipitate by the procedure of Rabinowitz et al. (20). For assay of radioactivity, duplicate 5-mg aliquots of each protein sample were dissolved in 1 ml of 1 M Hyamine-10X [7] solution by warming at 60° followed by addition of 9 ml of a solution of 0.5 diphenyloxazole in toluene. The samples were then counted in a Packard Tricarb liquid scintillation spectrometer. The counts were corrected for quenching by determining the recovery of an internal standard added to the sample.

TABLE 1

Composition of zinc-low basal diet

	g/100 g
Casein [1]	20.00
Cottonseed oil [2]	10.00
Vitamin-sucrose mix [3]	5.00
Mineral mix [4]	3.55
Choline chloride	0.15
Sucrose	61.30

[1] Nutritional Biochemicals Corporation. This was rendered low in zinc by a procedure combining isoelectric precipitation and chelation with ethylenediaminetetraacetic acid (EDTA).

[2] Wesson Oil, Wesson Oil Sales Company, Fullerton, California. Vitamin A, 1000 IU (Aquasol A, U. S. Vitamin and Pharmaceutical Corp., New York); vitamin D_2, 125 IU (Drisdol, Winthrop Laboratories, New York) and α-tocopheryl acetate, 60 mg (Nutritional Biochemicals Corp., Cleveland) were admixed with this oil.

[3] Vitamin-sucrose mix contained in mg/kg of mix: thiamine·HCl, 200; riboflavin, 120; pyridoxine·HCl, 80; Ca pantothenate, 320; biotin, 4; nicotinic acid, 300; folic acid, 10; vitamin B_{12}, 0.4; menadione, 6.6; sucrose added to make 1000 g.

[4] Each 3.55 g of mineral mix contained: (in grams) $CaHPO_4$, 2.58; KCl, 0.343; Na_2CO_3, 0.115; $MgSO_4 \cdot 7H_2O$, 0.40; $FeSO_4 \cdot 7H_2O$, 0.06; $MnSO_4 \cdot H_2O$, 0.031; $CoCl_2 \cdot 6H_2O$, 0.004; $CuSO_4 \cdot 5H_2O$, 0.006; KI, 0.0004; NaF, 0.0008.

The incorporation of adenine-8-^{14}C into nucleic acid was determined in aliquots of RNA and DNA extracts by following the method of Traketellis and Axelrod (21). Aliquots of the nucleic acid extracts were evaporated to dryness at 90° and the residue taken up in 2 ml of 1 M Hyamine-10X solution. The suspension was allowed to stand for 2 hours at room temperature with frequent stirring, the salt separated by centrifugation, and 1-ml aliquots of the supernatant were removed. To these aliquots, 9 ml of the 0.5% diphenyloxazole scintillation fluid was added and the solution was assayed for radioactivity by the same procedure used in the protein assay.

For histological examination, the testes were placed in Helly's solution for 3 hours, and a cross section (± 0.5 mm^3) was removed from the mid-portion and left in the fixative for 17 hours. These tissues were washed in tap water for 30 minutes, dehydrated in two changes of dioxane for 3 hours, and cleared in acetone for 1 hour. They were embedded in paraffin,[8] cut into 5-μ sections and stained with the periodic acid-Schiff procedure (22).

[5] "Purified" casein. Nutritional Biochemicals Corporation, Cleveland.

[6] Analytical Methods for Atomic Absorption Spectrophotometer Manual no. 990-9461. Perkin-Elmer Corporation, Norwalk, Connecticut, 1964.

[7] Hydroxide of Hyamine-10X. Packard Instrument Company, Downers Grove, Illinois.

[8] Paraplast, Fisher Scientific Company, New York.

RESULTS

In the first experiment, 24 21-day-old, weanling Sprague-Dawley rats (± 55 g) were fed the "low zinc diet." After 10 days, the rats had attained a mean weight of 81 g. They were then divided into 6 groups of 4 rats which consisted of duplicate zinc-deficient groups and corresponding pair-fed and ad libitum-fed control groups. The diet of both control groups (pair-fed and ad libitum) was supplemented with an additional 30 ppm of zinc added as $ZnSO_4 \cdot 7H_2O$. One zinc-deficient group and its control groups were killed after one week and the remaining groups were killed after 2 weeks of feeding the diets. The testes were rapidly removed and portions taken for histopathologic studies and for zinc analyses. The remainders of the organs were quickly frozen before analyses for DNA, RNA, protein, free amino acids and nucleotides.

The rats fed the deficient diet for 7 days weighed less than their controls, but had significantly heavier testes, 1588 mg vs. 1537 mg and 1517 mg, respectively (see table 2). At this time the testes of the deficient rats contained significantly less zinc and somewhat less protein than those of the pair-fed controls. Deficient rats killed on day 14 weighed considerably less and their testes were much smaller than those of their respective controls. The total zinc, RNA and protein content of the testes of deficient rats were significantly less but the total amounts of DNA in the testes of both groups were comparable on both days 7 and 14.

TABLE 2

Body and testes weights, and total content of zinc, DNA, RNA, and protein nitrogen in the testes of zinc-deficient and control rats [1,2]

Experimental day	Zinc-deficient		Pair-fed controls		Ad lib-fed controls	
Body wt, g						
0	81.7	81.0	81.5	80.8	81.0	79.8
7	89.1[a,b]		99.8		108.1	
14		92.7[a,b]		126.1		144.2
Difference [3]	+7.4	+11.7	+18.3	+45.3	+27.7	+64.4
Testes, mg						
7	1588 ± 18[a,b]		1537 ± 20		1517 ± 33	
14	1658 ± 32[a,b]		2094 ± 82		2145 ± 35	
Difference [3]	+70 (ns)		+557 ($P < 0.01$)		+628 ($P < 0.01$)	
Zinc, μg						
7	27.7 ± 0.96[a,b]		32.6 ± 0.62		32.8 ± 7.2	
14	28.7 ± 1.40[a,b]		48.2 ± 1.61		49.7 ± 12.6	
Difference [3]	+1.0 (ns)		+15.7 ($P < 0.001$)		+16.9 ($P < 0.001$)	
DNA, mg						
7	5.16 ± 0.25		5.26 ± 0.24		5.09 ± 0.15	
14	6.42 ± 0.21		6.69 ± 0.27		7.81 ± 0.31	
Difference [3]	+1.26($P < 0.05$)		+1.43 ($P < 0.05$)		+2.72 ($P < 0.05$)	
RNA, mg						
7	8.15 ± 0.25		8.32 ± 0.33		8.64 ± 0.28	
14	7.35 ± 0.21[a,b]		8.87 ± 0.32		9.12 ± 0.23	
Difference [3]	−0.80 (ns)		+0.55 (ns)		+0.48 (ns)	
Protein N, mg						
7	16.36 ± 0.61[b]		18.21 ± 1.24		18.75 ± 0.60	
14	18.60 ± 1.23[a,b]		24.91 ± 1.18		25.26 ± 1.10	
Difference [3]	+2.24 (ns)		+6.70 ($P < 0.05$)		+6.51 ($P < 0.05$)	

[1] Each dietary group consisted of two sub-groups of 4 rats each. One sub-group from each group was killed after 7 days of feeding and the other after 14 days.
[2] Values are means or means ± 1 SE.
[3] Day 7 minus day 0; or day 14 minus day 7 means. The statistical significance of the difference is indicated in parentheses; ns = not significant.
[a] Different from mean of pair-fed control ($P < 0.05$).
[b] Different from mean of ad lib-fed control ($P < 0.05$).

TABLE 3

Concentrations of different constituents of the testes of zinc-deficient and control rats [1,2]

Exp. group	Zinc	DNA	RNA	Protein N
	μg/g	*mg/g*	*mg/g*	*mg/g*
		Day 7		
Zn-deficient	17.5 ± 0.69 [a]	3.25 ± 0.18	5.14 ± 0.19 [b]	10.30 ± 0.27
Pair-fed controls	21.2 ± 0.34	3.43 ± 0.20	5.42 ± 0.29	11.83 ± 0.69
Ad lib-fed controls	21.6 ± 0.50	3.36 ± 0.27	5.69 ± 0.12	12.36 ± 0.33
		Day 14		
Zn-deficient	17.9 ± 0.38 [a]	3.89 ± 0.13 [a]	4.45 ± 0.14	11.23 ± 0.54
Pair-fed controls	23.0 ± 0.45	3.19 ± 0.08	4.19 ± 0.08	11.84 ± 0.20
Ad lib-fed controls	23.2 ± 0.39	3.64 ± 0.14	4.25 ± 0.09	11.77 ± 0.39

[1] Values are means ± 1 SE.
[2] Testes wet weight.
[a] Difference from mean of pair-fed controls is statistically significant ($P < 0.05$).
[b] Difference from mean of ad lib-fed control is statistically significant ($P < 0.05$).

A comparison of data obtained within groups on the seventh and fourteenth days shows that the only significant change in the deficient group was an increase in DNA. In the case of the pair-fed control group, all constituents measured increased significantly except RNA.

When a comparison is made on the basis of concentrations per gram of wet weight (table 3), the testes from the deficient rats killed on day 7 had zinc and protein concentrations that were significantly lower than those for both control groups. It will be remembered that at this time the testes from the deficient rats were actually larger than those from both control groups. This suggests accumulation of water had taken place. The apparent reduction in RNA concentration may also be explained similarly. Increase of another unmeasured tissue constituent might also be responsible but it appears unlikely. Lipid, the most obvious suspect, is present in small amounts in the testes and Bieri and Prival (23) have reported little change of this constituent in zinc-deficient rats.

On day 14, the testes of the deficient rats had significantly higher DNA concentrations but similar RNA and protein concentrations. This suggests that similar numbers of functioning cells are present but that protein synthesis has been reduced. The decrease in protein concentration observed in the ad libitum controls (7 days vs. 14 days) has been reported by others as a normal consequence of maturation (24).

When the concentrations of the various testicular constituents are compared using DNA as a base line (table 4), the zinc, RNA, and protein in the deficient testes were comparable on day 7, but significantly less than those of the pair-fed controls on day 14. Amino acid concentrations (expressed per mg protein nitrogen) were significantly elevated in the zinc-deficient animals on day 7, but the difference

TABLE 4

Zinc, RNA, protein nitrogen, TCA-soluble nucleotide concentrations and TCA-soluble amino acid concentrations in the testes of zinc-deficient and control rats

	Zinc, μg/mg DNA	
Zn-deficient	5.41 ± 0.36 [1]	4.62 ± 0.09[a,b]
Pair-fed controls	6.24 ± 0.32	7.23 ± 0.12
Ad lib-fed controls	6.46 ± 0.28	6.39 ± 0.50
	Protein N, mg/mg DNA	
Zn-deficient	3.20 ± 0.21 [b]	2.89 ± 0.13[a]
Pair-fed controls	3.50 ± 0.33	3.72 ± 0.05
Ad lib-fed controls	3.70 ± 0.19	3.23 ± 0.07
	RNA, mg/mg DNA	
Zn-deficient	1.59 ± 0.16	1.14 ± 0.005 [a]
Pair-fed controls	1.59 ± 0.09	1.31 ± 0.03
Ad lib-fed controls	1.70 ± 0.13	1.17 ± 0.02
	Amino acids, mg protein N	
Zn-deficient	2.66 ± 0.11[a,b]	2.33 ± 0.08
Pair-fed controls	2.19 ± 0.10	2.11 ± 0.07
Ad lib-fed controls	2.27 ± 0.09	2.11 ± 0.10

[1] Values are means ± 1 SE.
[a] Difference from mean of pair-fed controls is statistically significant ($P < 0.05$).
[b] Difference from mean of ad libitum-fed controls is statistically significant ($P < 0.05$).

obtained on day 14 did not attain statistical significance.

Cellular changes in the testes of the deficient and control groups were evident on day 14. On day 7, immature seminiferous tubules which were similar in morphology were present in the testes of both groups. Maturation of cells was observed in the testes examined on day 14. Many tubules in both groups were characterized by spermiogenesis and the presence of mature sperm. The presence of numerous sperm in the epididymal ducts of the control rats clearly indicated that maturation was attained. In the deficient testes, the tubules were smaller in diameter with spermatocytes and early spermatids present in some of the lumina. Immature, germinal epithelial cells were seen in the ducts of their epididymes as illustrated in figure 1. On day 14, testes from the deficient rats (with one exception) showed a marked loss of cells from the seminiferous tubules to the epididymes as a morphological variation from their controls.

In the second experiment, 22 weanling male rats were fed the low zinc diet (2.5 ppm) for 10 days. They were then divided into 3 groups. Two groups (8 and 6 rats, respectively) were fed the deficient diet. The third group (8 rats) was pair-fed the control diet to the deficient group of 8 rats. On day 13 of the study, all rats in the deficient group of six were repleted with 1 mg of zinc ($ZnSO_4 \cdot 7H_2O$) by intraperitoneal injection. This repletion regimen has been shown to initiate rapid regression of esophageal parakeratosis.[9] Twenty-four hours later, half of the animals in each group were injected intraperitoneally with L-leucine-U-^{14}C [10] and half were injected with adenine-8-^{14}C.[11] The isotopic dosage of both ^{14}C-leucine and adenine 8-^{14}C was 5 μCi per 100 g body weight. All rats were killed 4 hours after injection. The testes and spleens were removed and subjected to various analyses.

The mean body weights of these groups when killed (14–15 days) were 101 g (deficient group), 131 g (pair-fed group) and 89 g (zinc-repleted group). For reasons which were not apparent, the group destined for zinc repletion developed a more severe deficiency during the 2-week trial. When the animals were killed, the testes in this group were significantly smaller than those of the deficient, nonrepleted animals. These and other data are shown in table 5. Testes weights and concentrations of dry material, zinc, protein and RNA were lower in the deficient than in the pair-fed controls when expressed on a wet-weight basis. When protein and RNA concentrations were calculated on a dry-weight basis, the mean protein concentration, but not the RNA concentration of the deficient testes, was significantly lower than that in the pair-fed controls. Zinc concentrations in the testes of the repleted animals attained levels comparable to those of the controls and had higher concentrations of protein, which suggested that increased protein synthesis was induced by zinc repletion. It appears unlikely that it is due simply to a greater degree of dehydration because the concentration of dry materials did not differ from that of the pair-fed controls.

Despite this apparent decrease in protein concentration, the incorporation of radioactive leucine into proteins of testes from zinc-deficient rats did not differ significantly from that of the controls (table 6), nor did zinc repletion demonstrably effect the incorporation of leucine. The specific activities of testicular RNA and DNA and the ratios of these activities were comparable in all the groups.

DISCUSSION

The biochemical data suggest that no gross impairment of the synthesis of DNA exists in the testes early in zinc deficiency. This is indicated by the comparable amounts and specific activities of DNA found following administration of ^{14}C-adenine. The reports of Pelc (25) and Pelc and Howard (26) imply that it would have been more appropriate to have measured the specific activity of DNA 8 days after injection of the ^{14}C-adenine but, at this time, the rats would have been severely deficient.

In the first experiment in the deficient group there were slight decreases in pro-

[9] Unpublished results, G. H. Barney.

[10] L-Leucine-U-^{14}C, New England Nuclear Corporation, Boston; specific activity, 25.2 mCi/mmole. The solution injected was diluted with unlabeled leucine to give a specific activity of 25 μCi/mg.

[11] Adenine-8-C^{14}, New England Nuclear Corporation, Boston; specific activity, 4.22 mCi/mmole.

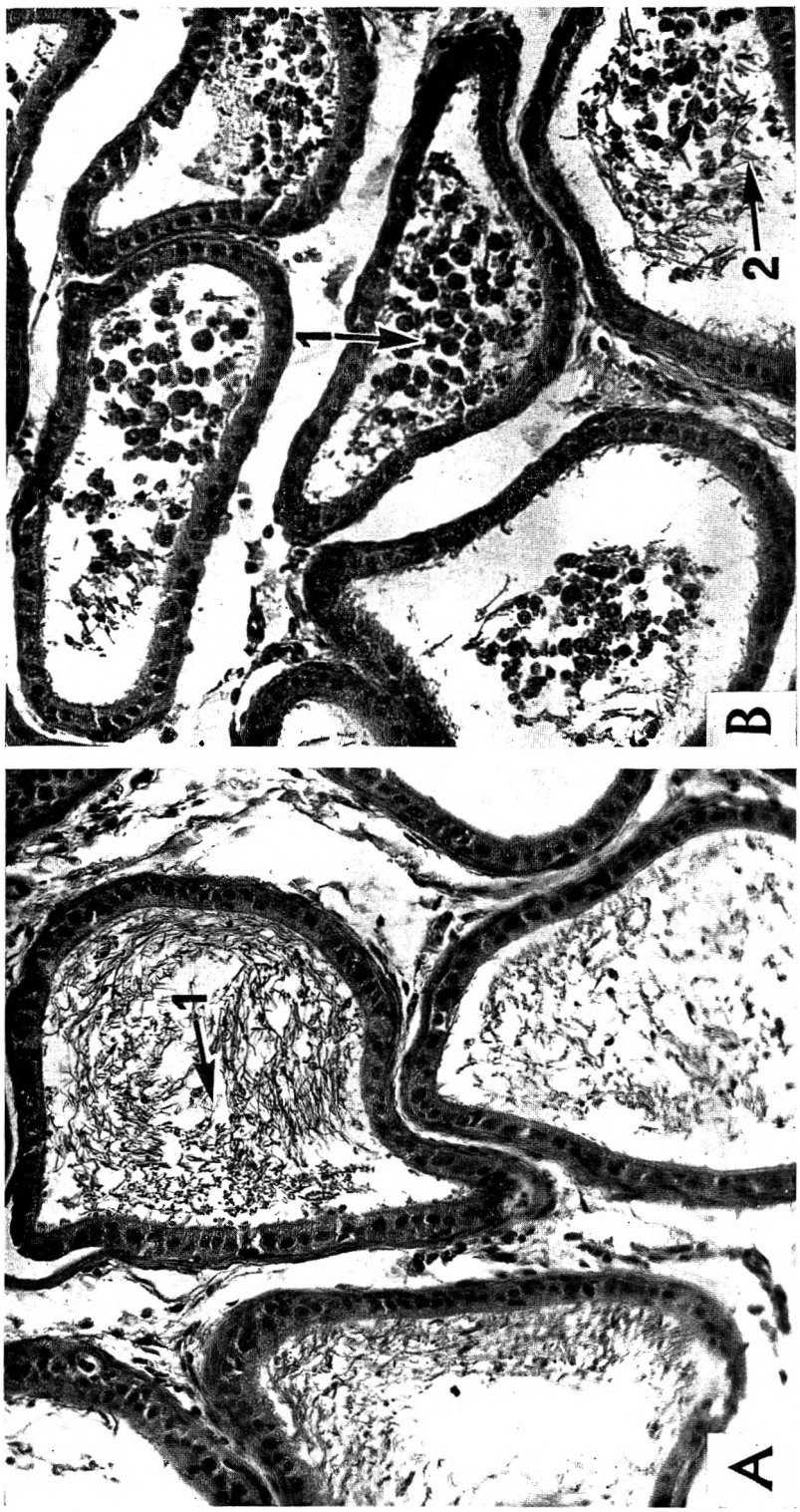

Fig. 1 (*A*) Photomicrograph of an epididymis from a zinc-supplemented, pair-fed rat. The ducts contain spermatozoa (1) that show normal morphology; (*B*) photomicrograph of an epididymis from a zinc-deficient rat illustrating numerous immature germinal epithelial cells (1) and a few spermatozoa (2) in the lumina of the ducts.

tein and RNA concentrations per milligram of DNA on day 14 of the experiment when compared with the pair-fed controls. Curiously, the latter group appeared to have an elevation of these values when compared with the ad libitum controls. Thus, on the basis of these data it does not seem appropriate to conclude that the synthesis of RNA and protein was depressed in the zinc-deficient rats. Rather, the inexplicable conclusion must be proffered that reduced feeding enhanced RNA and protein synthesis. In the second experiment, a similar trend was observed (data not presented), but since ad libitum-fed controls were not included, its significance is not clear.

TABLE 5

Concentrations of zinc, protein nitrogen, RNA, and DNA per gram wet weight of the testes of zinc-deficient, pair fed controls, and zinc-repleted deficient rats [1]

	Testes, wet wt *mg*	Protein N *mg/g wet wt*
Zn-deficient	$1879 \pm 47^{a,b}$	$12.50 \pm 0.23^{a,b}$
Pair-fed controls	2099 ± 54	13.40 ± 0.12
Repleted [2]	1638 ± 71^{a}	13.48 ± 0.19
	Dry wt, testes *mg/g wet wt*	RNA, *mg/g wet wt*
Zn-deficient	$126 \pm 2^{a,b}$	4.16 ± 0.05^{a}
Pair-fed controls	131 ± 1	4.34 ± 0.03
Repleted [2]	133 ± 2	4.37 ± 0.12
	Zinc concn *μg/g wet wt*	DNA, *mg/g wet wt*
Zn-deficient	$17.0 \pm 1.40^{a,b}$	3.77 ± 0.11
Pair-fed controls	24.5 ± 0.94	3.87 ± 0.16
Repleted [2]	23.2 ± 1.54	3.96 ± 0.15

[1] Values are means ± 1 SE; data from 8 zinc-deficient, 8 pair-fed controls, and 6 deficient rats that were repleted with zinc; total experimental period 14–15 days.
[2] Each animal received 1 mg zinc (as $ZnSO_4$) intraperitoneally 24 hours before killing.
[a] Difference from mean of pair-fed controls is statistically significant ($P < 0.05$).
[b] Difference from mean of repleted rats is statistically significant ($P < 0.05$).

Two other observations suggest that there was no reduction of RNA and protein synthesis in the zinc-deficient rats. In the first place, the rates of incorporation of leucine-U-^{14}C and adenine 8-^{14}C into protein and RNA, respectively, were not altered. Furthermore, although germinal cells progressively lose RNA during their differentiation into sperm (27, 28) and probably protein as well (29) normal differentiation into more mature cell types was apparently inhibited in the testes of zinc-deficient rats (fig. 1). Despite this, the percentage decrease in the RNA/DNA and protein/DNA ratios between days 7 and 14 in the deficient rats was only slightly less than that of the ad libitum controls. This implies that certain seminiferous tubules were affected and others were not, which is compatible with more comprehensive histological studies (30).

It is possible that these conflicting findings (i.e., protein and RNA reduced but leucine and adenine incorporation unaltered) are the result of an increase in the catabolism of protein and RNA rather than a decrease in synthesis. In starvation, increased breakdown of proteins takes place and increased tissue amino acid concentrations are observed (31). It is thus possible that the increase in amino acids (per mg protein N) observed initially in the testes may reflect increased breakdown of proteins even before manifest microscopic changes in the cells were seen. The report of Theuer and Hoekstra (10) who observed an increased rate of oxidation of intraperitoneally injected leucine-U-^{14}C to respiratory $^{14}CO_2$ in zinc-deficient rats, is compatible with the viewpoint.

The testicular zinc levels recorded here are somewhat lower than those reported

TABLE 6

Incorporation of leucine-U-^{14}C into proteins and of adenine-8-^{14}C into RNA and DNA of the testes of zinc-deficient, pair-fed controls and zinc-repleted deficient rats [1]

Exp. group	No. of animals	Proteins	DNA	RNA	Specific activity RNA/DNA
		cpm/mg	*cpm/mg*	*cpm/mg*	
Zn-deficient	4	269 ± 17	59 ± 14	384 ± 118	6.23 ± 0.81
Pair-fed controls	4	225 ± 8	59 ± 9	394 ± 80	6.47 ± 0.17
Repleted [2]	3	258 ± 16			

[1] Values are means ± 1 SE; animals received intraperitoneally, 5 μCi/100 g body weight of either leucine-U-^{14}C or adenine-8-^{14}C 4 hours before killing.
[2] Each animal received, intraperitoneally, 1 mg zinc (as $ZnSO_4$) 24 hours before killing.

by Prasad et al. (3) and by Parizek et al. (32). This may be explicable, in part, by the feeding of a low zinc diet in these studies before feeding the experimental diets. Although zinc has been shown to be present in high concentration (2 mg/g dry weight) in sperm (33), the physiological significance of this high concentration is unknown. The metal apparently is more concentrated in the tail portion (34) and is firmly bound (35). Wetterdale (36), in a serial examination of the incorporation of radioactivity in the testes and epididymes following the intramuscular injection of ^{65}Zn in the rat, concluded that zinc is "transported" with the sperm as they pass into the epididymes. According to the study of Gunn et al. (37), zinc in the supporting (non-tubular) structures of the testes is probably more exchangeable with the zinc of plasma.

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Linoleic Acid Requirement of the Hen for Reproduction

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ABSTRACT A study was designed to determine the linoleic acid (18:2) requirement of the hen for reproduction. Two hundred Leghorn pullets were reared and fed an essential fatty acid-deficient diet from 2 weeks of age through 22 weeks of age. At week 22, the depleted pullets were divided into 14 groups comprising duplicate pens of 7 different treatments, randomly arranged in a cage layer house. The groups were fed an 18:2 deficient diet supplemented with corn oil calculated on the basis of daily consumption to supply 0.0, 0.125, 0.25, 0.5, 1.0, 2.0, and 4% 18:2, respectively. All diets were isocaloric and isonitrogenous. After a 20-week experimental period, the data showed that the hen required approximately 2% dietary 18:2 for egg production and maximum egg size, and 1% 18:2 for hatchability of fertile eggs. The requirement for production of fertile eggs was less than 0.125%. Using the ratio of oleate to linoleate (18:1 to 18:2) in the plasma and liver as an estimation of dietary adequacy, the data illustrate that a ratio of 5.0 or less is indicative of an adequate intake of 18:2 for the hen. When 18:2 was expressed as percentage of dietary calories, the data showed the requirement for hatchability to be 3.14% and for egg production and maximum egg size, 6.19% of dietary calories. There were no significant differences in nutrient intake and body weights between each experimental group. Thus, the reproductive responses obtained were due to supplementary 18:2, and the requirement can be expressed as percentage of diet or as percentage of dietary calories.

Edwards (1) has suggested that the quantitative requirement of the hen for linoleic acid (18:2) is approximately 2.5%, but indicated that this estimate was based on results of experiments which were not primarily designed as requirement studies (2–5). Miller et al. (6) reported that a daily intake of 250 mg of polyunsaturated fatty acids was not adequate to meet the essential fatty acid requirement of the hen for maximal egg production. Later, Menge et al. (7) demonstrated that a daily intake of only 20 mg of 18:2 was sufficient for optimal fertility and embryonic viability through day 7 of incubation.

Since there are no clearly defined studies on the need of the hen for 18:2 for reproduction, the present study was designed to determine this requirement.

EXPERIMENTAL PROCEDURE

Two hundred White Leghorn pullets were fed a corn-soy diet from hatch until 2 weeks of age and then a linoleic acid-deficient grower diet (table 1) from 2 weeks until 22 weeks of age. This period of depletion was found to be sufficient to deplete the pullets of 18:2 (7). Further evidence of this depletion is seen in the response to supplemental 18:2. At week 22 the pullets were divided into 14 groups of 14 birds each on the basis of body weight and placed into individual laying cages. The 14 groups comprising duplicate pens of 7 different treatments were randomly arranged in a cage layer house. The 7 groups were fed the linoleic acid-deficient (0.002% 18:2) layer diet (table 1) supplemented with corn oil,[1] calculated to supply 0.0, 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0% 18:2, respectively. All diets were made isocaloric and isonitrogenous by adjustment of cellulose and carbohydrate. Feed weighbacks were made every 2 weeks, and the average individual daily consumption was calculated to determine the quantity of corn oil necessary to add to the diets of each group for the following 2 weeks to give the required dietary level of 18:2. The actual average daily consumption of 18:2 per hen for the 20-week

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[1] Supplied by Procter and Gamble Company, Cincinnati. The fatty acid composition of the crude corn oil as determined by gas-liquid chromatography was as follows: (percentage of total fatty acids) 16:0, 11.58; 16:1, 0.27; 18:0, 1.78; 18:1, 27.22; 18:2, 58.89; 18:3, 0.27.

TABLE 1

Composition of linoleic acid-deficient diet

Ingredient	Grower diet	Layer diet
	%	%
Casein (vitamin-free) [1]	18.00	—
Gelatin	11.00	—
Isolated soy protein [2]	—	19.00
Powdered cellulose [3]	2.00	5.00
Hydrogenated coconut oil [4]	2.00	1.00
Antioxidant [5]	0.015	0.015
Vitamin mix	0.5 [6]	0.5 [7]
Mineral mix	7.5 [8]	11.4 [9]
Methionine hydroxy analogue	0.34	0.4
Choline (70%)	0.35	0.35
Glucose monohydrate	58.295	62.335

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Assay protein C-1, Skidmore Enterprises, Cincinnati. This material was blended with powdered cellulose and extracted exhaustively with hot methanol.
[3] Solka Floc, BW-40, Brown Company, Berlin, New Hampshire.
[4] Supplied by the Procter and Gamble Company, Cincinnati.
[5] 1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinolone. Monsanto Company, St. Louis.
[6] Vitamin mix supplied the following: (mg/kg diet) thiamine·HCl, 100; riboflavin, 16; Ca D-pantothenate, 25; pyridoxine·HCl, 10; folacin, 4; Klotogen F, 15; niacin, 100; biotin, 0.6; vitamin B_{12} (0.1%), 20; vitamin A (500,000 USP/g), 36; vitamin D_3 (200,000 ICU/g), 7; *d*-α-tocopheryl acetate (1360 IU/g), 26; and glucose monohydrate to 0.5% of diet.
[7] Vitamin mix supplied the following: (mg/kg diet) thiamine·HCl, 50; riboflavin, 16; Ca D-pantothenate, 25; pyridoxine·HCl, 10; folacin, 5; Klotogen F, 15; niacin, 50; biotin, 0.5; vitamin B_{12} (0.1%), 15; dry vitamin A palmitate (500,000 USP/g), 36; D-activated sterol vitamin D_3 (200,000 ICU/g), 8; *d*-α-tocopheryl acetate (1360 IU/g), 40; and glucose monohydrate to 0.5% of diet.
[8] Mineral mix supplied the following: (in percent) $CaCO_3$, 2; $CaHPO_4 \cdot 2H_2O$, 1.3; KH_2PO_4, 1.4; NaCl, 0.64; $MgCO_3$, 1.75; $FeC_6H_5O_7 \cdot 5H_2O$, 0.06; $MnSO_4 \cdot H_2O$, 0.03; $ZnCO_3$, 0.01; KI, 0.0003; $Cu(CH_3COO)_2 \cdot H_2O$, 0.0032; H_3BO_3, 0.001; $CoSO_4 \cdot 7H_2O$, 0.0002; $Na_2MoO_4 \cdot 2H_2O$, 0.0009; $Na_2SeO_4 \cdot 10H_2O$, 0.00005; and glucose monohydrate to 7.5%.
[9] Mineral mix supplied the following: (in percent) $CaCO_3$, 5.2; $CaHPO_4 \cdot 2H_2O$, 4.1; KH_2PO_4, 1.2; $MgCO_3$, 0.425; NaCl, 0.3; $FeC_6H_5O_7 \cdot 5H_2O$, 0.06; $MnSO_4 \cdot H_2O$, 0.03; $ZnCO_3$, 0.0125; $Cu(CH_3COO)_2 \cdot H_2O$, 0.004; KI, 0.00015; H_3BO_3, 0.001; $CoSO_4 \cdot 7H_2O$, 0.0002; $Na_2MoO_4 \cdot H_2O$, 0.0006; $Na_2SeO_4 \cdot 10H_2O$, 0.000015; and glucose monohydrate to 11.4%.

experimental period was (in percent) 0.0, 0.127, 0.25, 0.44, 0.99, 1.96, and 3.89, respectively. Although some groups did not receive exactly the prescribed amounts of 18:2, the discrepancies were minimal.

When the average egg weight from the groups supplemented with 1% 18:2 reached 45 g, the pullets were artificially inseminated weekly with 0.05 ml pooled semen from Leghorn cocks. Individual body weights were recorded monthly, and egg production was tabulated daily. Eggs were stored at 13°, weighed collectively by pens at the end of each week and incubated. After 6 days of incubation, they were candled to determine fertility. Hatchability data were obtained at the end of the incubation period. The experiment was concluded after 20 weeks of collecting data on reproduction. The data were analyzed using least-squares procedures as described by Harvey (8). Duncan's multiple range test was applied to the least-squares means (9).

Four individual samples of plasma and liver were taken from randomly selected pullets in each experimental group at the termination of the experiment. The preparation of the lipid for fatty acid analysis was described by Miller et al. (6), and the fatty acid composition determined by the procedure outlined in a previous report (10).

RESULTS AND DISCUSSION

The data in table 2 illustrate the effect of different levels of 18:2 supplied by corn oil on the reproductive performance of essential fatty acid-depleted hens. The

TABLE 2

Effect of linoleic acid from corn oil on reproduction through a 20-week experimental period [1]

Treatment	Egg production	Egg wt	Fertile eggs	Viable germs [2]	Hatch of fertile eggs
Linoleic acid, %	%	*g*	%	%	%
0.0	61.8 [a]	44.9 [a]	90.4 [abc]	84.2 [a]	1.8 [a]
0.125	69.1 [b]	48.2 [a]	91.0 [abc]	91.4 [b]	49.0 [b]
0.25	68.9 [b]	49.9 [a]	88.0 [a]	91.2 [b]	73.1 [c]
0.5	72.0 [bc]	50.4 [a]	90.5 [abc]	93.1 [b]	83.4 [d]
1.0	75.0 [bc]	52.4 [b]	93.0 [c]	93.5 [b]	88.2 [e]
2.0	76.7 [cd]	53.5 [c]	90.1 [ab]	93.5 [b]	89.0 [e]
4.0	81.9 [d]	52.5 [b]	92.1 [bc]	93.9 [b]	89.4 [e]

[1] Means with different superscripts are significantly different at the 1% level according to Duncan's multiple range test (9).
[2] Percentage of total number of fertiles containing live embryos at day 7.

addition of 18:2 to the diet stimulated increases in egg production, egg weight, embryonic viability, and hatchability of fertile eggs, but had no effect on fertility.

Dietary 18:2 stimulated egg production with each increase in daily intake. The 18:2 requirement for egg production was not clearly defined in this study, but the data suggest that it is between 1 and 2% with this type of diet. This estimation of the 18:2 requirement for production agrees to some extent with the value of 2.5% reported from other laboratories (1, 5). According to the data in table 2, the 18.2 requirement of the hen for maximal egg size is approximately 2%. This value agrees with the results of Shutze et al. (2) in which they reported maximal egg size with 2.5% dietary 18:2, with no further increase in egg size with levels up to and including 7.5%. In agreement with earlier studies in this laboratory (7, 12), only small quantities of 18:2 are necessary for the production of fertile eggs containing viable embryos through day 7 of incubation. The results of the present study show that 0.125% 18:2 was sufficient for this reproductive characteristic.

The percentage hatch of fertile eggs appears to give the most sensitive response to 18:2. The lipid-containing materials in the diet must be exhaustively extracted to obtain zero hatchability (7, 11, 12). Small residues of 18:2 resulted in percentage hatchability of 1.2% (13) and 1.8% in the present study.

The data in table 2 place the 18:2 requirement of the hen for hatchability of fertile eggs at approximately 1% of the diet.

There was a gradual increase in the efficiency of egg production (food consumed per dozen eggs produced) as the level of 18:2 was increased (table 3). There were no significant differences in feed consumption and average body weights between experimental groups. A significant difference in nutrient intake and average body weights was found in an earlier study (13) in which a casein gelatin diet was used for an experimental period of 32 weeks. The results obtained in the present work show that the extracted isolated soy protein diet is quite satisfactory. Bray (14) has reported that controlled caloric intake could either negate or reduce the effect of dietary corn oil on egg weight depending upon the technique employed for controlling the intake. In the present study, the results show that increased levels of 18:2 (corn oil) in the diet of the hen stimulated an increase in egg size, even though there were no significant differences in consumption of the isocaloric, isonitrogenous diets or in body weights (tables 2 and 3). These results show that the increased egg weight was due to dietary 18:2, in agreement with the data reported by Shutze and Jensen (4).

Holman (15) has shown that linoleate, expressed as percentage of total dietary calories, is related to the ratio of eicosatrienoic to arachidonic (20:3 to 20:4) in the tissues of rats, and has also shown that this ratio is indicative of the essential fatty acid status of the animal. These

TABLE 3

Effect of linoleic acid (corn oil) on feed consumption, feed efficiency and body weight [1]

Treatment	Feed consumption [2]	Feed per dozen eggs	Body wt [3]
Linoleic acid, %	*g*	*kg*	*g*
0.0	84.3	1.78 [b]	1,550
0.125	92.9	1.78 [b]	1,590
0.25	93.3	1.81 [b]	1,640
0.5	89.5	1.63 [ab]	1,610
1.0	93.8	1.70 [ab]	1,610
2.0	92.7	1.60 [ab]	1,640
4.0	92.9	1.49 [a]	1,620

[1] Means with different superscripts are significantly different at the 1% level according to Duncan's multiple range test (9).
[2] Average feed consumed per hen per day for 20-week experimental period.
[3] Average body weight at end of experiment (42 weeks).

criteria have been used by others to determine the 18:2 requirement of swine (16–18), the guinea pig (19), and the chick (20–22). The application of these techniques to the results of the present study shows that the 18:2 requirement, expressed as percentage of dietary calories, is 3.14% for hatchability, and 6.19% for egg production and maximum egg size (table 4). The similarity of caloric intake for all experimental groups indicates that caloric intake is not necessarily related to dietary 18:2. Thus, the 18:2 requirement of the hen may be expressed equally well as percentage of diet or as percentage of dietary calories.

The ratios of 20:3 to 20:4 found in the plasma and liver lipids from the experimental groups are shown in table 4. These ratios decreased as the levels of dietary 18:2 were increased. This decrease reflected the change in the fatty acid profile from that of an essential fatty acid-depleted hen to a nondepleted hen. Although others have used this ratio to advantage, we have been unable to obtain a numerical ratio in the groups receiving from 1% to 4% 18:2 in the present study. Since only trace amounts of 20:3 were detectable in these groups with our chromatograph, it was impossible to assign a definite value. This would suggest that the 20:3-to-20:4 ratio has only limited application and cannot be correlated with reproductive performance since no values could be assigned to correspond with hatchability at the 1% level, and egg production and maximum egg size at the 2% level of 18:2.

Reid et al. (19) have suggested that the ratio of oleate to linoleate (18:1 to 18:2) may be a more valid estimation of dietary 18:2 adequacy than the 20:3-to-20:4 ratio. The present study suggests an additional advantage of the 18:1-to-18:2 ratio. The data presented in table 4 show values of 1.0 or more for 18:1-to-18:2 ratios, in contrast to values approaching zero for the 20:3-to-20:4 ratios in the experimental groups receiving 1% or more dietary 18:2. The 18:1-to-18:2 ratios exhibited the alleviation of the deficiency of essential fatty acids by a gradual decrease as the percentage 18:1 declined and the 18:2 increased in the tissues of hens receiving higher levels of 18:2. According to the data in table 4, an 18:1-to-18:2 ratio of 5.0 or less indicates an adequate intake of 18:2 for the hen for all reproductive functions.

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TABLE 4

The effect of different levels of dietary linoleic acid (corn oil) on the monoene-to-diene, and triene-to-tetraene ratios of plasma and liver lipids from hens [1]

Treatment	Linoleic as calories consumed [2]	Plasma		Liver	
		18:1 [3] to 18:2 [4]	20:3 [5] to 20:4 [6]	18:1 [3] to 18:2 [4]	20:3 [5] to 20:4 [6]
Linoleic acid, %	%				
0.0	0.0	76.3	8.3	90.4	5.3
0.125	0.40	35.7	2.4	21.5	1.0
0.25	0.79	40.7	1.0	27.7	0.9
0.50	1.39	13.7	0.4	12.5	0.2
1.0	3.14	5.1	tr [7]	5.8	tr [7]
2.0	6.19	2.5	tr [7]	2.8	tr [7]
4.0	12.27	1.3	tr [7]	1.0	tr [7]

[1] Samples taken after a 20-week experimental period.
[2] Average daily consumption of linoleic acid (9 kcal/g) calories/285 kcal metabolizable energy/100 g diet.
[3] Monoene (oleic acid).
[4] Diene (linoleic acid).
[5] Triene (tentatively identified as 5, 8, 11 isomer of eicosatrienoic acid).
[6] Tetraene (arachidonic acid).
[7] Only trace amounts of 20:3 were detectable.

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Effect of Positional Distribution on the Absorption of the Fatty Acids of Human Milk and Infant Formulas [1]

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ABSTRACT A fat absorption study was conducted in rats to determine if the unusually high proportion of palmitic acid in the 2-position of triglycerides could be related to the superior absorption of human milk fat. Digestion yields free fatty acids and 2-monoglycerides. The monoglycerides of the saturated fatty acids are more readily absorbed than the free acids. Tests run on a number of fats commonly fed to infants, showed that the high absorption of human milk fat, 94.6 (%), was equaled by a fat blend of a similar fatty acid composition with a high content of 2-palmitoyltriglyceride, 96.3, in contrast to one with low content, 89.9. Absorption of butterfat was 89.5, lard 92.4 and oleo 79.9. Individual fatty acid absorption was influenced by total fat absorption. A linear relationship between absorption and the proportion in the 2 position was demonstrated for palmitic, and to a lesser extent, for myristic and oleic, but not for stearic acid. Equations predicting fat absorption required emphasis of the product of stearic acid and palmitic acid esterified in the primary positions.

The fat constituent of the milk formulas fed to most bottle-fed infants in this country is butterfat, or mixtures of vegetable or vegetable and animal fats, often formulated to simulate the composition of human milk fat. While these fats are well absorbed, most clinical studies have shown human milk fat to be better absorbed, particularly in premature and newborn infants (1–6).

In a comprehensive study of fat absorption in infants conducted over 30 years ago, Holt and his co-workers (1) concluded that the absorption of a fat could be predicted from its fatty acid composition. Unsaturated or short-chain fatty acids favored absorption; long-chain saturated fatty acids impaired it. Thus, while the superior absorption of the fat of human milk over that of a cow's milk formula can be explained by the higher degree of unsaturation of the fat, and to a lesser extent by the lower mineral content (1), this explanation does not hold for current commercial formulas of low mineral content and closely similar fatty acid composition.

Another possibility relates to an unusual feature of the glyceride structure of human milk fat; a relatively high proportion of the palmitic acid is in the 2 position of the triglyceride molecule (7). This positional distribution may confer a nutritional advantage. Pancreatic lipase specifically attacks the primary ester linkages (8) with the result that a large proportion of the palmitic acid is present in the intestinal tract as a monoglyceride. The monoglycerides of the less well-absorbed fatty acids are believed to be better absorbed than the free acids (9). Thus, the absorption of palmitic acid may be greater from human milk fat than from any fat in which it is esterified predominantly in the 1,3 positions.

It was the purpose of the present study to examine the relationship between fat absorption in the rat and the composition and positional distribution of the fatty acids of the fats and oils commonly used in infant feeding.

MATERIALS AND METHODS

Fat samples. Human milk fat was prepared by extracting a lyophilized composite sample of mature human milk with chloroform-methanol (2:1) and removing the

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solvent by distillation. The butter, lard, oleo oil and vegetable oils used in the following mixtures were commercial samples. The composition of these blends was: (in per cent) OSCC — 40 oleo oil, 25 soybean oil, 12 corn oil and 23 coconut oil; OPPC — 25 oleo oil, 20 peanut oil, 35 palm oil and 20 coconut oil; LBC — 70 lard, 20 butter fat and 10 coconut oil. The OPPC and the LBC mixtures were formulated to give a fatty acid composition very similar to that of human milk. The pertinent difference between the two was that the LBC mixture, like human milk fat, had a high proportion of palmitic acid in the 2 position while in the OPPC mixture palmitic was esterified predominantly in the 1,3 positions. The lard component of the LBC blend supplied the high content of 2-palmitoyltriglyceride (10–11). The OSCC blend contained less palmitic and more linoleic acid than human milk fat. Lard and oleo were selected for comparison because, although they were quite similar in content of palmitic and oleic acids, they differed markedly in the proportion of palmitic acid in the 2 position.

Positional distribution of the fatty acids. The proportion of each fatty acid in the 2 position of the triglycerides was determined by the procedure of Brockerhoff and Yurkowski (12). The fat samples were digested by pancreatic lipase to a predetermined end point and the monoglyceride fraction was isolated by thin-layer chromatography on silica gel G plates developed with 1.5% acetic acid in isopropyl ether. The fatty acid composition of the original triglyceride and of the 2 monoglycerides was determined by gas-liquid chromatography.

Fat absorption procedure. Young rats of the Sprague-Dawley strain were fed a fat-free basal diet consisting of: (g/kg) casein, 220; glucose, 738; salt mixture, 40(13); vitamin mixture, 1(13); and choline chloride 1. Each rat received a daily supplement of 0.1 ml of corn oil except during a test and 3 days prior to it.

The rats were divided into groups with equal average weight, 6 rats/group. Each group was fed the test fat incorporated into the basal diet at the 15% level replacing an equal amount of glucose. This diet was fed ad libitum for 3 days, followed by the fat-free diet for 3 more days. A preliminary study had shown that fat excretion returns to the endogenous level within this period. Feces were collected for the 6-day period and stored under ethanol. In each experiment a group receiving only the fat-free diet for 6 days served as a control for the estimation of the endogenous fecal fat excretion for rats of this age and body weight. The total fatty acid content of the food and feces was determined by the method of van de Kamer (14), and an aliquot of the isolated fatty acid fractions was analyzed by gas-liquid chromatography. The absorptions of the total and individual fatty acids were calculated from the ratios of the amounts in the feces corrected for endogenous excretion to the amounts ingested.

Samples for gas-liquid chromatography were methylated by the procedure of Metcalfe et al. (15). The esters were separated on a 183 cm × 0.635 cm column packed with 10% ethylenesuccinate methylsilicone and Gas Chrom P.[2] The separation of the various peaks conformed to the requirements recommended by the American Oil Chemists Society (16).

EXPERIMENTAL RESULTS

Positional distribution of fatty acids. The results of analysis of the fat samples for fatty acid composition and the proportions of each of the major fatty acids in the 2 position of the triglycerides are presented in table 1. The proportions of the fatty acids found in the 2 position are in good agreement with published values for human milk fat, butter, lard and oleo oil (beef fat) (7, 17–19) and the values for the experimental mixtures are consistent with reported data on the constituent oils (20).

A comparison of the values for palmitic, stearic, and oleic acids shows that the LBC and OPPC mixtures duplicate the total fatty acid composition of human milk fat reasonably well, but only the LBC mixture resembles human milk fat in the high proportion of palmitic acid in the 2 position (table 2). The proportion of 2-palmitic acid is high in lard, intermediate in butter fat, and low in oleo, OPPC

[2] Applied Science Laboratories, State College, Pennsylvania.

TABLE 1

Composition of fat samples and the proportion of fatty acids in the 2 position

Fatty acids	LBC [1]		Human milk		OPPC [2]		OSCC [3]		Lard		Oleo		Butterfat	
	% [4]	%β [5]	%	%β	%	%β	%	%β	%	%β	%	%β	%	%β
C12:0	5.3	67	4.7	36	10.0	73	11.4	74	—	—	—	—	2.9	51
C14:0	5.1	62	7.8	57	5.0	34	5.6	34	1.6	88	3.6	67	12.0	69
C16:0	25.2	71	27.3	68	25.8	18	17.1	13	28.8	84	26.3	18	30.7	43
C18:0	11.6	11	9.7	5	7.7	12	9.1	12	13.0	10	18.6	14	11.6	11
C18:1	38.9	7	34.5	9	35.6	35	27.4	32	43.3	8	43.6	44	26.0	16
C18:2	8.7	12	9.5	18	11.4	51	23.8	36	11.2	10	2.4	44	1.8	18
C18:3	0.8	—	1.5	—	0.7	—	3.0	26	1.0	—	—	—	0.9	—

[1] LBC — lard, butter and coconut (see text for proportions).
[2] OPPC — oleo, palm, peanut and coconut.
[3] OSCC — oleo, soybean, corn and coconut.
[4] By weight.
[5] Percent in 2 position.

TABLE 2

Fatty acid composition of the feces [1,2]

Dietary fat	LBC [3]	Human milk	OPPC [3]	OSCC [3]	Lard	Oleo oil	Butterfat	Fat-free
				weight %				
C12:0	1.3	1.3	3.8	3.6	—	—	1.0	0.2
C14:0	3.7	4.2	6.1	6.2	0.7	2.0	7.9	1.5
C16:0	29.3	30.2	52.2	41.9	23.4	41.8	50.5	21.0
C18:0	44.1	37.0	22.3	30.5	53.3	41.6	29.3	16.2
C18:1	14.0	19.6	10.2	12.5	17.6	10.8	7.9	11.4
C18:2	1.4	1.5	1.0	2.4	1.5	0.3	0.2	2.4
C18:3	0.8	1.7	—	—	1.3	0.3	—	7.4
Others	5.4	4.5	4.1	2.9	3.2	0.2	3.2	39.9

[1] Average values for 2–5 groups of rats, 6/group.
[2] Uncorrected for endogenous excretion.
[3] See footnote to table 1.

and OSCC. Stearic acid is predominantly present in the 1,3 position in all fats. The distribution of oleic acid varied from 7 to 44% in the 2 position.

Fatty acid composition of the feces. The fatty acid composition of the feces of rats fed the various fats is presented in table 2. Stearic, palmitic, and oleic acids constituted 85 to 94% of the fatty acids of the feces from rats fed fats. In contrast, 40% of the fecal fatty acids of rats fed the fat-free diet consisted of fatty acids other than the common food fatty acids. In addition to those listed in the table, 17 other fatty acids were found; predominant among these were C15:0(9%), C20:0(3%), C20:4(3%), C22:0(4%), C24:0(8%), and C24:1(3%). The nature of the "endogenous" fecal fat has been reported to be determined by the intestinal bacteria and not to vary with the type of fat in the diet, or the depot fat of the rats (21). In the current study it was assumed that the endogenous fat excretion did not change when fat was ingested. Results consonant with this assumption were obtained in a preliminary experiment in which excretion was measured in groups of rats fed diets with the same fat varying in content from 5 to 30% of the diet. When the regression line of fecal fat to food fat was extrapolated to zero fat intake, the calculated value did not differ appreciably from that obtained with the feeding of a fat-free diet.

Fat absorption studies. The detailed data from a typical test are presented in table 3. Table 4 summarizes the results of all assays, with the absorbability of the various fats presented in decreasing order. Since the replicate determinations agreed closely, the values for each fat were combined for statistical analysis. With the exception of the comparisons, OSCC versus lard and OPPC versus butter, each average

TABLE 3

Data from a typical fat absorption experiment

Fat	Intake	Excreted	Absorption [1]	
	mmoles	*mmoles*	%	
Human	26.25	1.21	92.7	
	31.05	1.91	94.4	
	21.40	1.89	91.2	94.7 ± 0.90
	27.80	1.32	96.3	
	25.80	1.15	97.1	
	27.50	1.33	96.3	
LBC [2]	22.15	0.85	97.5	
	26.10	1.38	95.9	
	27.75	1.74	94.8	
	32.25	2.31	93.8	95.9 ± 0.55
	31.00	1.35	96.7	
	27.30	1.32	96.4	
Oleo oil	34.10	7.04	80.5	
	29.60	6.97	77.5	
	29.35	6.45	79.0	78.6 ± 0.49
	28.40	6.64	77.7	
	30.35	7.09	77.6	
	26.55	5.84	79.1	
Lard	27.95	2.77	91.2	
	31.05	2.37	93.3	
	29.00	3.00	90.7	92.5 ± 0.66
	19.85	1.99	91.5	
	32.25	2.16	94.2	
	28.15	1.89	94.4	
Fat-free		0.31		
		0.32		
		0.33		
		0.28		
		0.26		
		0.32		
		0.30 Avg.		

[1] Corrected for endogenous fat excretion.
[2] LBC — lard, butter and coconut (see text for proportions).

TABLE 4

Total fatty acid absorption

Fat	Absorption determinations				Avg ± SE
	1	2	3	4	
	%	%	%	%	
LBC [1]	95.9 ± 0.55	96.6 ± 0.70	—	—	96.3 ± 0.46
Human milk fat	94.8 ± 0.35	94.8 ± 0.83	95.3 ± 0.30	94.7 ± 0.90	94.9 ± 0.14
OSCC [1]	92.2 ± 0.90	95.7 ± 0.57	94.4 ± 0.66	—	93.4 ± 0.41
Lard	92.3 ± 0.67	92.5 ± 0.66	—	—	92.4 ± 0.45
OPPC [1]	90.6 ± 0.72	89.1 ± 0.74	90.1 ± 0.28	—	89.9 ± 0.17
Butterfat	90.4 ± 1.3	88.7 ± 0.41	—	—	89.5 ± 0.85
Oleo oil	81.2 ± 1.9	78.6 ± 0.49	—	—	79.9 ± 0.93

[1] See footnote to table 1.

value was significantly different from all the others (t test, $P = 0.05$ or less).

The LBC mixture, resembling human milk fat in total fatty acid composition but containing a slightly higher proportion of 2-palmitic acid, was slightly better absorbed than human milk fat. In contrast, the OPPC mixture also with a fatty acid composition simulating human milk but with a low proportion of 2-palmitic acid

was definitely less well absorbed. Lard was more efficiently absorbed than oleo; however, superior absorption cannot be entirely attributed to the differences in content of 2-palmitic acid. Although the two fats are reasonably alike in total fatty acid composition, lard contains less stearic and more linoleic acid than oleo, both factors favoring total absorption.

The absorptions of the individual fatty acids in each fat are presented in table 5. The ranking of the fatty acids was the same for all fats; linoleic acid was the best absorbed, followed in order by lauric or oleic, myristic, palmitic and stearic acids.

The data of figure 1 show that the absorption of each fatty acid is in general directly related to the absorption of the total fatty acids of the fat; i.e., in a less well absorbed fat, such as oleo, every fatty acid is less well absorbed than in the better absorbed fats. Figure 1 also shows that some fatty acids deviate from the general trend; in lard, palmitic acid is exceptionally well absorbed, but it is relatively poorly absorbed in OSCC and OPPC.

This influence of the total fatty acid composition on the absorption of the individual fatty acid tends to obscure correlations between positional distribution and the absorption of the fatty acid. In the data of figure 2 the effect of positional distribution on absorption has been isolated from the numerous other variables by expressing the absorption of the fatty acid as the ratio of its absorption to the absorption of the total fatty acids on the particu-

TABLE 5

Absorption of individual fatty acids

Fatty acid	LBC	Human milk	OSCC [1]	Lard	OPPC [1]	Butterfat	Oleo oil
				absorption, %			
C12:0	99.4	98.7	97.5	—	96.1	96.5	—
C14:0	97.1	96.8	91.5	98.0	87.8	92.7	89.1
C16:0	95.1	92.5	84.2	93.4	78.2	82.5	67.0
C18:0	82.3	74.3	74.2	68.0	70.4	73.4	53.4
C18:1	98.6	97.1	97.7	96.8	97.1	96.9	94.1
C18:2	99.5	99.4	99.4	99.4	99.1	99.5	98.3
Total fatty acids	96.3	94.9	93.4	92.4	89.9	89.5	79.9

[1] See footnote to table 1.

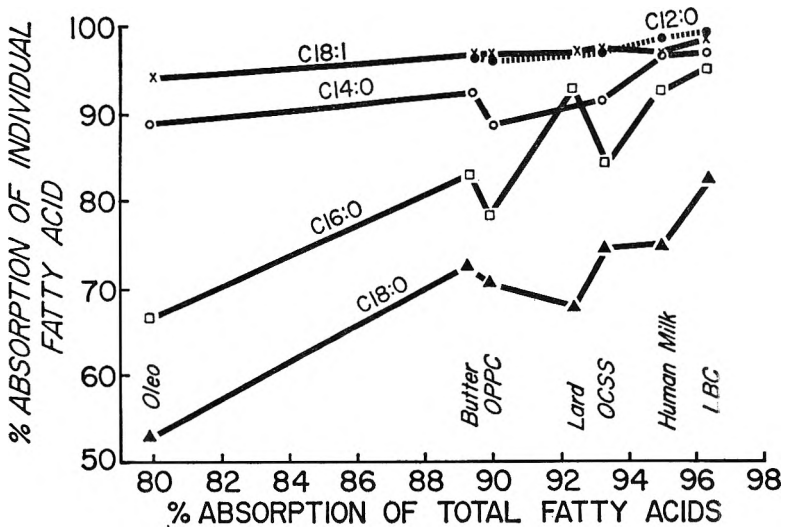

Fig. 1 Relationship of the absorption of the individual fatty acids to total fatty acids.

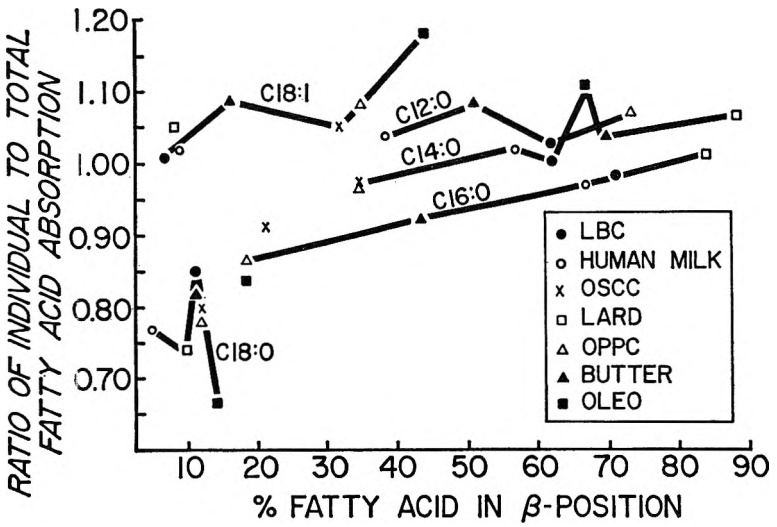

Fig. 2 Relative fatty acid absorption and the proportion in the β-(2) position.

lar fat. The data of figure 2 clearly indicate that the relative absorption of palmitic acid was linearly related to the proportion in the 2 position of the triglyceride molecule. This relationship, with several exceptions, was also true for oleic acid and myristic acids. The positional distribution on the glyceride molecule did not appear to influence the absorption of lauric acid or stearic acid. In the case of stearic acid, however, this conclusion can only be tentative since, in the fat samples tested, stearic acid was predominantly in the 1,3 positions.

DISCUSSION

The results of the present study assessing the influence of positional distribution of the fatty acids on fat absorption have offered an explanation for the superior absorption of human milk fat by the infant that could not be explained by fatty acid composition alone. The importance of the positional distribution of the fatty acids of the common food fats applies particularly to palmitic acid since it is one of the major constituents and is relatively poorly absorbed. The unsaturated and the short-chain saturated fatty acids are well absorbed regardless of positional distribution. Stearic acid, the most poorly absorbed of the common fatty acids, is generally present at concentrations only one-half to one-third that of palmitic acid, and in all fats tested to date (7, 17–20) was predominantly in the primary positions. In the present study the absorption of stearic acid could not be related to the proportion in the 2 position suggesting that the monoglyceride of stearic acid is no better absorbed than the free acid. As mentioned previously, however, the percent in the 2 position varied only from 5 to 14% in the seven fats studied and the influence of other factors may have overridden that of positional distribution.

The present results, indicating a relationship between the positional distribution and the absorption of palmitic acid, are in accord with the results of the chick study conducted by Renner and Hill (22). These authors found that when lard is "randomized," i.e., treated so that the palmitic and stearic acids are equally distributed on the glyceride molecule, there was a significant loss in the absorption of palmitic acid consistent with the decrease in the proportion in the 2 position. Stearic acid absorption was found to increase slightly, but the increase was not statistically significant. Under the adverse influence of decreased palmitic absorption, however, a concomitant decrease in stearic absorption would be expected, so that the slight increase found was not inconsistent with an improvement in absorption result-

ing from an increase in the proportion of 2 esterified stearic acid from 10 to 33%. The relationship of absorption and positional distribution of stearic acid will require further study.

In analyzing the data of the present study it appeared that, at least with the seven fats studied, the most important factors adversely influencing total fat absorption in the rat are the content of palmitic acid in the 1,3 positions, and the total stearic acid content. With the aid of a digital computer numerous equations associating fat excretion with fatty acid composition were examined and a high degree of correlation was found in all equations emphasizing the content of 1,3-palmitic and 1,3- or total stearic acid. An example of one of the simpler descriptions of the relationship was:

total fatty acid excreted = 0.05 (1,3-palmitic × 1,3-stearic) + 2.5; r^2 = 0.91; with fatty acid concentrations expressed as mole percent.

In studies with the chick, Young and Garrett (23) also noted an interaction between stearic and palmitic acids, which when fed together tended to decrease the absorption of each other.

No convincing evidence was found in the current study that either oleic or linoleic acid specifically influenced the absorption of palmitic or stearic acids. Common food fat mixtures of high unsaturation are necessarily low in long-chain saturated fatty acids, and the improvement in stearic or palmitic absorption can be explained as readily by the decreased product of the interaction between these two acids as by the increase in unsaturation. Experiments are now in progress with fat blends of a composition more appropriate for the study of the influence of unsaturated and short-chain fatty acids on palmitic and stearic acid absorption.

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Dietary Regulation of Pyruvate Kinase Synthesis in Rat Liver [1]

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ABSTRACT The effects of diet and antibiotics on pyruvate kinase, glucose 6-phosphatase and tyrosine-α-ketoglutarate transaminase activities were investigated in male Sprague-Dawley rats. Feeding a 90% casein diet to animals pre-fed with a 90% carbohydrate diet for 4 days increased the activities of all 3 enzymes. Force-feeding with casein hydrolysate increased glucose 6-phosphatase, tyrosine-α-ketoglutarate transaminase and pyruvate kinase activity, though the latter increase was observable only in the animals pre-fed with glucose. The increase in glucose 6-phosphatase activity was partially prevented by actinomycin D and almost totally prevented by cycloheximide. The increase in pyruvate kinase activity (in the animals pre-fed with glucose) was not affected by actinomycin D, but was prevented by cycloheximide. Both antibiotics increased tyrosine-α-ketoglutarate transaminase activity far beyond the increase caused by force-feeding casein hydrolysate alone. 8-Azaguanine decreased the induction of glucose 6-phosphatase by force-feeding casein hydrolysate without inducing tyrosine-α-ketoglutarate transaminase or decreasing the induction of the transaminase by actinomycin D or cyclohexamide. Pyruvate kinase activity was induced by force-feeding carbohydrate to rats pre-fed for 5 days with a 90% casein diet. These increases were prevented by both actinomycin D and cycloheximide. Tyrosine-α-ketoglutarate transaminase was not induced by the antibiotics in these animals. Generally, enzyme activities were higher in the animals pre-fed with fructose, even after force-feeding. The results are in agreement with those for an earlier proposed mechanism of control for the synthesis of pyruvate kinase; that is, the synthesis of this enzyme is controlled at the translational level when the dietary change is from a protein-free to a high protein diet and at the transcriptional level in the case of the high protein to protein-free dietary change.

In previous reports (1, 2) it was noted that pyruvate kinase activity can be increased by a high protein diet following pre-feeding a high carbohydrate diet or by feeding a high carbohydrate regimen to rats pre-fed a high protein diet (3). Since carbohydrate has been shown to be a potent inducer of pyruvate kinase (4, 5), it was suggested (1, 2) that pyruvate kinase activity was increased by a high protein diet following the feeding of a protein-free, high carbohydrate regimen by relieving the inhibition of protein synthesis brought about by the absence of protein in the pre-fed diet. Furthermore, it was suggested (1, 2) that the transitory increase in pyruvate kinase activity after the feeding of a high protein diet was due to increased synthesis of the enzyme regulated at the level of translation and, therefore, should be independent of messenger RNA synthesis. However, the induction of pyruvate kinase by high carbohydrate diet following the feeding of a high protein diet was postulated to involve increased synthesis of messenger RNA (1–3) and should be dependent on messenger RNA synthesis. These experiments were undertaken to test the assumptions above, using diets and antibiotics.

Preliminary work showed that tyrosine-α-ketoglutarate transaminase activity was increased by actinomycin D and cycloheximide. Therefore, the relationship between dietary treatment, antibiotics and tyrosine-α-ketoglutarate transaminase activity was also investigated.

EXPERIMENTAL

Animals. Male rats of the Sprague-Dawley strain, weighing 160 to 190 g were used for all experiments. The animals

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were housed individually in screen-bottom cages and were offered water ad libitum.

To establish a uniform dietary background, the animals were fed for 3 to 5 days a commercial laboratory ration immediately after receipt. Two basic experimental designs were used. The first experimental design was the following: The animals were fed ad libitum for 4 days a diet consisting of 5% corn oil; 4% minerals (6); 1% vitamins (7); and 90% carbohydrate, either glucose, or fructose. The control animals were killed after 4 days of being fed the 90% carbohydrate diet. The experimental animals were subjected to further dietary treatment: One group was force-fed twice with 4 ml/feeding of 50% casein hydrolysate (enzymatically hydrolysed), first at 10 AM and again at 10 PM, another group was fed the same amount of casein hydrolysate (4 g of powder without corn oil, minerals or vitamins) ad libitum and the third group was fed ad libitum the 90% casein diet containing corn oil, salt and vitamins in the same proportion as described above. The experimental animals were killed 24 hours after the first force-feeding or dietary change.

The second experimental design was the following: The animals were fed ad libitum for 5 days the 90% casein diet. In the control group, food was withdrawn 12 hours before killing; in the experimental groups, food was withdrawn for the same length of time before the dietary change. The experimental animals were subjected to the following dietary treatments: One group was fed ad libitum a 90% carbohydrate diet (glucose or fructose) for 1 or 2 days before killing, and another group was force-fed 3 times (10 AM, 4 PM and 10 PM) with 4 ml/feeding of 50% glucose or sucrose. The force-fed animals were killed 24 hours after the first force-feeding.

Antibiotics were injected intraperitoneally in 2 doses, one at 10 AM and the second at 10 PM. The injections were administered immediately after force-feeding. A single dose was administered in a volume of 1.5 ml of water. 8-Azaguanine was dissolved by adjusting the pH of the solution to ten. Solutions containing actinomycin D [2] and 8-azaguanine were prepared by first dissolving actinomycin D in water and then adding 8-azaguanine and adjusting to pH 10. When cycloheximide and 8-azaguanine were both administered, they were injected separately in a total volume of 1.75 ml.

Determinations. Animals were decapitated in the early morning. The livers were immediately removed, rinsed, blotted, weighed and chilled over ice. A 10% liver homogenate was prepared with ice-cold, 0.14 M KCl (pH 7.4) using a Potter-Elvehjem homogenizer. Total liver protein was determined in this crude homogenate by a modified biuret procedure (8). Liver glycogen was determined by a turbidimetric method (9). The crude homogenate was centrifuged at zero to 4° for 30 minutes at 20,000 × *g* and the clear homogenate was used for the determination of soluble liver protein (8), pyruvate kinase (10) and tyrosine-α-ketoglutarate transaminase (11). The latter 2 enzymes were assayed at 25° using a Gilford Model 2000 multiple absorbance recorder. Glucose 6-phosphatase activity was determined by measuring the amount of inorganic phosphate liberated in 15 minutes at 37° using a 5% crude liver homogenate prepared with ice-cold, 0.1 M potassium citrate buffer (pH 6.5) (12). Enzyme activity was expressed as units per 100 g body weight and one unit of enzyme activity was defined as the amount of enzyme which can produce 1 μmole of product/minute under the conditions of the assay.

RESULTS

Effects of dietary treatment on body size, relative liver size and liver protein. Body size was decreased 14 to 18% by feeding the protein-free, high carbohydrate diets for 4 days. The weight loss was about the same whether the carbohydrate fed was glucose or fructose. Body size was slightly increased by feeding the 90% casein diet for 5 days.

Relative liver size (RLS) [3] values were 4.33 in the animals fed the commercial laboratory ration, 3.81 in the animals pre-fed with the 90% glucose diet, 5.09 in the animals pre-fed with the 90% fructose diet

[2] Actinomycin D was a generous gift from the Merck, Sharp and Dohme Company.

[3] RLS = (weight of liver) × 100/(body weight at time of killing).

and 3.95 after feeding the 90% casein diet for 5 days (table 1). Since body size was decreased about equally by either of the 2 carbohydrate diets fed, the large values of RLS observed in the animals fed fructose can be attributed to a better maintenance of liver weight.

The effects of refeeding with the 90% casein diet after feeding the high carbohydrate diets for 4 days was dependent on the carbohydrate fed: thus, in the animals pre-fed with the 90% glucose diet, RLS was increased by protein feeding, though slightly, whereas in the fructose-fed animals protein feeding caused a very substantial decrease in RLS (table 1). However, the feeding of casein hydrolysate, especially when force-fed, caused a substantial reduction in RLS (tables 1, 2), even in the animals pre-fed with glucose. This may have been due to induced intestinal stress or inadequate absorption from the intestine. Diarrhea observed in rats force-fed with casein hydrolysate, especially when injected with antibiotics, indicated that force-feeding with casein hydrolysate and injection of antibiotics did produce a certain amount of intestinal trauma.

In the animals maintained with the commercial laboratory ration, total liver protein values averaged 905 mg/100 g body weight. This value was decreased by feeding the 90% glucose diet to 716 (table 1). In contrast with this, the feeding of the 90% fructose diet (table 1) caused no detectable decreases in liver protein values. It is significant that liver protein values remained high in fructose-fed animals even though the diet contained no protein, whereas this was not true if glucose were fed. The reasons for this difference are not clear.

The feeding of casein hydrolysate (force-fed or fed ad libitum) to rats pre-fed with the 90% glucose diet did not restore total liver protein (tables 1, 2). The 90% casein diet, however, increased total liver protein values to the levels found in animals maintained on a commercial laboratory preparation (table 1). In the animals pre-fed with fructose the feeding of casein hydrolysate or the 90% casein diet caused a slight decrease in total protein. The 20% decrease in RLS values, observed in the fructose-fed rats after protein feedings, therefore, must have occurred mostly at the expense of liver components other than protein.

Changes in liver glycogen due to diet and antibiotics. Liver glycogen values are given as milligrams per 100 g body weight. From these values the glycogen content of liver in per cent can be calculated by dividing the milligrams/100 g body weight values by 10 times the RLS.

TABLE 1

Liver constituents in rats fed ad libitum

Dietary treatment	Relative liver size [1]	Liver glycogen	Total liver protein
		mg/100 g body wt	*mg/100 g body wt*
Laboratory ration [2] (8) [3]	4.33 ± 0.12 [4]	168 ± 13	905 ± 14
4 days 90% glucose (8)	3.81 ± 0.17	191 ± 24	716 ± 21
+1 day 90% casein (8)	4.09 ± 0.08	37.8 ± 6.1	928 ± 52
+1 day casein hydrolysate [5] (8)	3.48 ± 0.07	< 10	652 ± 21
4 days 90% fructose (10)	5.09 ± 0.15	393 ± 33	936 ± 27
+1 day 90% casein (8)	4.09 ± 0.08	< 10	861 ± 34
+1 day casein hydrolysate [5] (8)	3.65 ± 0.07	< 10	684 ± 36
5 days 90% casein [6] (8)	3.95 ± 0.09	39.7 ± 3.2	914 ± 45
+1 day 90% glucose [6] (4)	4.18 ± 0.19	269 ± 43	954 ± 10
+2 days 90% glucose [6] (2)	3.87	161	585
+1 day 90% fructose [6] (4)	4.03 ± 0.17	147 ± 94	928 ± 89
+2 days 90% fructose [6] (2)	4.48	200	748

[1] (Weight of liver × 100)/body weight.
[2] Pelleted Purina Rat Chow, Ralston Purina Company, St. Louis.
[3] Number of animals used in experiment.
[4] SE of mean.
[5] All the animals were offered 4 g of casein hydrolysate; some animals consumed less than this amount.
[6] The 90% casein diet was withdrawn for 12 hours before killing or further dietary treatment.

Liver glycogen values were (in mg/100 g body wt and per cent, respectively) 168 (3.88%) in rats fed the commercial preparation, 191 (5.02%) in glucose-fed rats, 393 (7.73%) in fructose-fed rats, and 39.7 (1.05%) in rats fed the 90% casein diet for 5 days (table 1). Force-feeding of casein hydrolysate or feeding an equal amount of casein hydrolysate ad libitum decreased liver glycogen to nonmeasurable levels (tables 1, 2). Feeding the 90% casein diet to these animals caused an almost equal depletion (table 1). The sharp decrease in liver glycogen values were not caused by force-feeding, as when glucose or sucrose were force-fed to rats adapted to a 90% casein diet; the glycogen content of liver was substantially increased (table 3). It appears then that increased glycogenolysis is an important part of the stress syndrome which is brought about by a sudden absence of carbohydrate from the diet and the subsequent influx of amino acids into the liver. The increase in glycogen deposition produced by force-feeding carbohydrate was reduced, but not eliminated, by antibiotics (table 3). This indicates that the antibiotics also cause a certain amount of stress, but not nearly as much as occurs due to the force-feeding of casein hydrolysate. The inhibitory effect of antibiotics on glycogen deposition after force-feeding carbohydrate is qualitatively similar to the partial inhibition of glycogen deposition by actinomycin D following the injection of glucocorticoids (13, 14).

Induction of glucose 6-phosphatase. The activity of glucose 6-phosphatase in animals fed the commercially prepared diet was found to be about 55 units/100 g body weight (table 4). This activity was not reduced by feeding the 90% glucose diet for 4 days (table 4). Casein hydrolysate, force-fed or fed ad libitum, increased this value by about 100% (tables 4, 5). Feeding the high protein diet to the animals pre-fed with glucose produced about the same amount of increase in glucose 6-phosphatase activity. Part of the induction produced by force-feeding casein hydrolysate was inhibited by actinomycin D and cycloheximide (table 5), the latter being more effective in preventing the increase in the activity of this enzyme. Previously, the induction of glucose 6-phosphatase by cortisol was shown to be prevented by actinomycin D (15), ethionine (16) and 8-azaguanine (17). The in-

TABLE 2

Liver constituents in rats force-fed with casein hydrolysate

Dietary treatment	Injections [1]	Relative liver size [2]	Liver glycogen	Total liver protein
			mg/100 g body wt	*mg/100 g body wt*
		Pre-fed 4 days with 90% glucose diet		
Control (8) [3]	none	3.81 ± 0.17 [4]	191 ±24	716 ± 21
Force-fed [5] (4)	PSP [6]	3.90 ± 0.16	< 20	653 ± 72
Force-fed (5)	2 × 15 μg AcD	3.79 ± 0.13	< 10	809 ± 60
Force-fed (3)	2 × 25 μg CHX	3.13 ± 0.08	< 10	577 ± 85
		Pre-fed 4 days with 90% fructose diet		
Control (10)	none	5.09 ± 0.15	393 ±33	936 ± 27
Force-fed [5] (8)	PSP	4.12 ± 0.08	< 10	810 ± 61
Force-fed (6)	2 × 7.5 mg 8AG	4.13 ± 0.14	33.4 ± 7.5	759 ± 39
Force-fed (12)	2 × 15 μg AcD	4.00 ± 0.08	< 10	759 ± 43
Force-fed (8)	2 × 25 μg CHX	3.89 ± 0.13	< 10	707 ± 53
Force-fed (11)	2 × (15 μg AcD + 7.5 mg 8AG)	3.95 ± 0.04	< 10	833 ± 95
Force-fed (11)	2 × (25 μg CHX + 7.5 mg 8AG)	3.95 ± 0.18	< 10	738 ± 81

[1] Injections were given after each force-feeding.
[2] (Liver weight × 100)/body weight.
[3] Number of animals used in experiment.
[4] SE of mean.
[5] Rats were force-fed at 10 AM and 10 PM. Each rat was force-fed 4 ml of warm 50% casein hydrolysate per force-feeding. The animals were killed 24 hours after the first force-feeding.
[6] Abbreviations used: PSP = physiological saline; AcD = actinomycin D; CHX = cycloheximide and 8AG = 8-azaguanine.

TABLE 3

Liver constituents in rats force-fed with carbohydrates

Dietary treatment	Injections [1]	Relative liver size [2]	Liver glycogen	Total liver protein
			mg/100 g body wt	*mg/100 g body wt*
Control [3] (8) [4]	none	3.95 ± 0.09 [5]	39.7 ± 3.2	914 ± 45
Force-fed,[6] glucose (10)	PSP [7]	3.75 ± 0.10	130 ± 14	774 ± 33
Force-fed, glucose (10)	2 × 15 μg AcD	3.66 ± 0.09	58.1 ± 16	701 ± 24
Force-fed, glucose (10)	2 × 25 μg CHX	4.02 ± 0.10	39.2 ± 14	799 ± 35
Force-fed, sucrose (6)	PSP	3.78 ± 0.14	95.5 ± 18	806 ± 10
Force-fed, sucrose (8)	2 × 15 μg AcD	3.75 ± 0.10	81.9 ± 15	720 ± 23
Force-fed, sucrose (8)	2 × 25 μg CHX	4.22 ± 0.09	58.4 ± 15	802 ± 26

[1] Injections were given after force-feeding at 10 AM and at 10 PM.
[2] (Liver weight × 100)/body weight.
[3] All the animals were fed for 5 days the 90% casein diet. Twelve hours before killing (control) or before force-feeding, the diet was withdrawn.
[4] Number of animals used in experiment.
[5] SE of mean.
[6] Rats were force-fed at 10 AM, 4 PM and 10 PM. Each rat was force-fed 4 ml of warm 50% carbohydrate solution per force-feeding. The animals were killed 24 hours after the first force-feeding.
[7] Abbreviations used: PSP = physiological saline; AcD = actinomycin D and CHX = cycloheximide.

TABLE 4

Enzyme activities in rats fed ad libitum

Dietary treatment	Glucose 6-phosphatase	Pyruvate kinase	Tyrosine-α-ketoglutarate transaminase
	μmoles/min/100 g body wt		
Laboratory ration [1] (8) [2]	55.6 ± 3.3 [3]	80.4 ± 5.9	2.06 ± 0.21
4 days 90% glucose (8)	56.2 ± 2.9	89.4 ± 5.6	1.49 ± 0.28
+1 day 90% casein (8)	91.9 ± 5.9	134 ± 15	10.6 ± 0.99
+1 day casein hydrolysate [4] (8)	116 ± 3.2	96.1 ± 6.0	3.97 ± 0.51
4 days 90% fructose (10)	80.4 ± 3.9	133 ± 3.8	1.91 ± 0.24
+1 day 90% casein (8)	122 ± 5.0	171 ± 10	10.6 ± 2.1
+1 day casein hydrolysate [4] (8)	121 ± 11	125 ± 16	5.49 ± 1.5
5 days 90% casein [5] (8)	64.0 ± 3.0	48.4 ± 3.0	2.87 ± 1.1
+1 day 90% glucose [5] (4)	53.0 ± 12	85.9 ± 6.8	— [6]
+2 days 90% glucose [5] (2)	58.3	93.2	— [6]
+1 day 90% fructose [5] (4)	81.5 ± 4.8	111 ± 5.4	— [6]
+2 days 90% fructose [5] (2)	87.8	155	— [6]

[1] Pelleted Purina Rat Chow, Ralston Purina Company, St. Louis.
[2] Number of animals used in experiment.
[3] SE of mean.
[4] All animals were offered 4 g of casein hydrolysate; some animals consumed less than this amount.
[5] The 90% casein diet was withdrawn 12 hours before killing or further dietary treatment.
[6] Values not determined.

duction of this enzyme in response to feeding a high fructose diet was shown to be inhibited by ethionine (18).

Fructose-feeding (table 4) increased glucose 6-phosphatase activity by about 40% above values observed in animals fed the commercial laboratory ration. In the fructose-fed animals casein hydrolysate or the 90% casein diet produced a further induction (table 5). This induction, observed in rats force-fed with casein hydrolysate, was partially inhibited by actinomycin D and cycloheximide and completely inhibited by 8-azaguanine given alone or in combination with one of the other 2 antibiotics (table 5). The dosage of 8-azaguanine was far in excess of the amount of the other 2 antibiotics given, because actinomycin D and cycloheximide are very toxic, even at these concentrations, whereas the animals can tolerate 8-azaguanine in high concentrations for days.

Glucose 6-phosphatase was also increased by feeding 90% casein diet for 5 days (table 4), though to a very small ex-

TABLE 5

Enzyme activities in rats force-fed with casein hydrolysate

Dietary treatment	Injections [1]	Glucose 6-phosphatase	Pyruvate kinase	Tyrosine-α-ketoglutarate transaminase
		μmoles/min/100 g body wt		
Pre-fed 4 days with 90% glucose diet				
Control (8) [2]	none	56.2± 2.9 [3]	89.4± 5.6	1.49±0.28
Force-fed [4] (4)	PSP [5]	100± 5.1	101± 5.3	2.86±0.40
Force-fed (5)	2×15 μg AcD	80.4± 5.3	115±14	9.79±3.1
Force-fed (3)	2×25 μg CHX	73.0± 9.3	80.0± 6.7	11.3 ±0.78
Pre-fed 4 days with 90% fructose diet				
Control (10)	none	80.4± 3.9	133± 3.8	1.91±0.24
Force-fed [4] (8)	PSP	111± 6.1	146± 5.5	4.39±0.77
Force-fed (6)	2×7.5 mg 8AG	77.9± 2.0	190± 8.0	4.83±0.67
Force-fed (12)	2×15 μg AcD	97.3± 3.4	149±12	15.2 ±1.8
Force-fed (8)	2×25 μg CHX	95.0± 5.2	149±14	12.9 ±0.40
Force-fed (11)	2×(15 μg AcD+7.5 mg 8AG)	82.7±13	143± 5.8	11.2 ±1.8
Force-fed (11)	2×(25 μg CHX+7.5 mg 8AG)	79.0± 4.2	136± 7.6	14.1 ±2.1

[1] Injections were given after each force-feeding.
[2] Number of animals used in experiment.
[3] SE of mean.
[4] Rats were force-fed at 10 AM and 10 PM. Each rat was force-fed 4 ml of warm 50% casein hydrolysate solution per force-feeding. The animals were killed 24 hours after the first force-feeding.
[5] Abbreviations used: PSP = physiological saline; AcD = actinomycin D; CHX = cycloheximide and 8AG = 8-azaguanine.

TABLE 6

Enzyme activities in rats force-fed with carbohydrates

Dietary treatment	Injections [1]	Glucose 6-phosphatase	Pyruvate kinase	Tyrosine-α-ketoglutarate transaminase
		μmoles/min/100 g body wt		
Control [2] (8) [3]	none	64.0± 3.5 [4]	48.4±3.0	2.87±1.1
Force-fed,[5] glucose (10)	PSP [6]	53.0± 3.5	70.0±1.1	1.00±0.13
Force-fed, glucose (10)	2×15 μg AcD	55.6± 3.0	54.8±3.6	1.65±0.11
Force-fed, glucose (10)	2×25 μg CHX	72.7±10	41.2±3.0	1.93±0.24
Force-fed, sucrose (6)	PSP	61.9± 4.9	94.9±8.4	0.96±0.07
Force-fed, sucrose (8)	2×15 μg AcD	58.1± 5.6	69.4±3.5	1.92±0.25
Force-fed, sucrose (8)	2×25 μg CHX	67.3± 5.7	54.2±4.2	1.83±0.24

[1] Injections were given after force-feeding at 10 AM and 10 PM.
[2] All the animals were fed for five days the 90% casein diet. Twelve hours before killing (control) or force-feeding, the diet was withdrawn.
[3] Number of animals used in experiment.
[4] SE of mean.
[5] Rats were force-fed at 10 AM, 4 PM and 10 PM. Each rat was force-fed 4 ml of 50% warm carbohydrate solution per force-feeding. The animals were killed 24 hours after the first force-feeding.
[6] Abbreviations used PSP = physiological saline; AcD = actinomycin D and CHX = cycloheximide.

tent. This is in agreement with earlier reports that as the animals adapt to a high protein diet the activity of liver glucose 6-phosphatase begins to approach pre-induction levels (1, 2). Force-feeding with glucose (table 6) or feeding a 90% glucose diet ad libitum (table 4) decreased the activity of this enzyme to the level observed in the animals fed the commercial preparation. However, force-feeding of sucrose (table 6) did not produce such decreases, whereas ad libitum feeding of the 90% fructose diet (table 4) increased glucose 6-phosphatase activity. Furthermore, when force-feeding of carbohydrates was combined with the injection of antibiotics (table 6) a slight, but noticeable increase in the activity of this enzyme was observed. Such increases, however, may have been due to the conversion of the enzyme to a more active form, rather than *de novo* synthesis (19).

Translational control of pyruvate kinase synthesis. It has been reported that py-

ruvate kinase activity can be increased by a 90% casein diet following the feeding of a 90% glucose diet for 4 days (1). In another report, it was noted that pyruvate kinase activity was increased by a 90% glucose or fructose diet following pre-feeding for 5 days with a 90% casein diet (3). In all these cases the increase in pyruvate kinase activity was temporary, lasting for 1, 2 or at most 3 days after the dietary change. Since dietary carbohydrate has been shown to induce pyruvate kinase (4) and a high protein diet depresses the activity of this enzyme (5), the induction of pyruvate kinase by a 90% casein diet after feeding a 90% glucose diet for 4 days was unexpected. To reconcile this finding with the work of others, it was proposed that the high carbohydrate diet induces the synthesis of a messenger RNA specific for pyruvate kinase, but the synthesis of the enzyme is not increased because of a general or specific inhibition of protein synthesis due to the lack of protein in the diet. When the 90% casein diet is fed to these animals, the inhibition of synthesis is relieved, the rate of enzyme synthesis is increased and pyruvate kinase activity increases. This increase, however, is transitory, because the synthesis of specific messenger RNA is reduced by the high protein diet and pyruvate kinase activity decreases as the specific messenger RNA decays. If the dietary shift is reversed, pre-feeding with the 90% casein diet results in low pyruvate kinase activity, because of a reduced amount of specific messenger RNA. Feeding the high carbohydrate, protein-free diet to these animals will again increase pyruvate kinase activity because of an increase in the synthesis of specific messenger RNA, and the increase in enzyme activity will persist until the reduction of synthesis at the translational level is established due to the lack of protein in the diet.

To test the validity of this proposal, the following reasoning was used: 1. Pyruvate kinase activity should be inducible by the 90% casein diet after feeding a 90% carbohydrate diet for 4 days. 2. This induction should occur whether the pre-fed carbohydrate was glucose of fructose, though the base level and the induced level should be higher in the animals pre-fed the fructose. 3. Pyruvate kinase should be inducible by force-feeding casein hydrolysate to animals pre-fed with a 90% carbohydrate diet. 4. The induction should be inhibited by cycloheximide, but not by actinomycin D, since *de novo* synthesis of messenger RNA is not required. 5. Pyruvate kinase should be inducible by feeding a high carbohydrate diet to rats pre-fed for 5 days with a high protein, carbohydrate-free diet. 6. Pyruvate kinase activity in these animals should also be induced by force-feeding carbohydrate. 7. The induction produced by force-feeding carbohydrate should be inhibited by cycloheximide and actinomycin D since this induction was proposed to involve an increased synthesis of protein and messenger RNA.

Most of the results are in agreement with the proposed mechanism of regulation of pyruvate kinase synthesis: 1. The activity of pyruvate kinase was induced by feeding a 90% casein diet to rats pre-fed with a 90% glucose diet for 4 days (table 4). 2. This induction also occurred in rats pre-fed with fructose and the pre-induction and induced levels of pyruvate kinase activity were both higher than the corresponding values in the animals pre-fed with glucose (table 4). 3. Pyruvate kinase activity was induced by force-feeding casein hydrolysate to rats pre-fed with glucose but not in rats pre-fed with fructose (table 5). 4. The induction of pyruvate kinase in the animals pre-fed with glucose was inhibited by cycloheximide, but not by actinomycin D (table 5). 5. Pyruvate kinase activity was low following the feeding of 90% casein diet for 5 days and was increased by feeding the 90% carbohydrate diets ad libitum (table 4). 6. Pyruvate kinase activity was increased by force-feeding carbohydrates (table 6). 7. The increase in pyruvate kinase activity produced by force-feeding carbohydrates was inhibited by actinomycin D and cycloheximide (table 6). It appears then that pyruvate kinase synthesis can be regulated both at the transcriptional and translational levels. The only piece of evidence which did not support this hypothesis was the failure of casein hydrolysate to increase pyruvate kinase activity in the animals pre-fed with fructose. However, pyruvate

kinase activity was already high in the fructose-fed animals and the activity of this enzyme was increased by feeding the 90% casein diet. The failure of casein hydrolysate to increase pyruvate kinase activity in these animals, then, may have simply been due to the fact that the animals were fed only 4 g of casein hydrolysate as compared with the 10 to 14 g of casein diet eaten by the animals fed ad libitum.

Superinduction and control of tyrosine-α-ketoglutarate transaminase synthesis. Tyrosine-α-ketoglutarate transaminase activity has been reported to be inducible by glucocorticoids (20, 21), pancreatic hormones (22–24), force-feeding with casein hydrolysate (21), dietary protein (1, 2, 25), actinomycin D (26, 27), and cycloheximide (28). The activity of the enzyme is believed to be regulated by a repressor system, that is, tyrosine-α-ketoglutarate transaminase synthesis is normally repressed (29).

Evidence for a repressor system of tyrosine-α-ketoglutarate transaminase synthesis was first found by Garren and co-workers (26). Their findings were recently confirmed (27). According to these authors (26, 27), the induction of tyrosine-α-ketoglutarate transaminase proceeds up to 4 hours when the production of a repressor is induced. Injection of actinomycin D before the onset of repressor induction will prolong the increase in tyrosine-α-ketoglutarate transaminase activity (which normally returns to pre-induction levels 7 hours after induction); if the antibiotic is injected after the repressor is induced, the decline in tyrosine-α-ketoglutarate transaminase activity is not prevented (27). The reported induction of tyrosine-α-ketoglutarate transaminase by puromycin (27), cycloheximide (28), and a contradiction of the cycloheximide findings (30), have caused considerable controversy regarding the induction of tyrosine-α-ketoglutarate transaminase.

In these experiments tyrosine-α-ketoglutarate transaminase activity was found to be dependent on diet and the nature of antibiotic used. The activity of the transaminase in the animals maintained with the commercially prepared laboratory ration, the glucose-fed animals and the fructose-fed animals (table 4) varied between 1.49 and 2.06 μmoles/min/100 g body weight. Casein hydrolysate (fed ad libitum or force-fed) produced a twofold increase in the animals pre-fed with glucose and a somewhat higher increase in the animals pre-fed with fructose (tables 4, 5). Tyrosine-α-ketoglutarate transaminase activity was increased about fivefold by feeding the high protein diet irrespective of the carbohydrate used in pre-feeding (table 4). Enzyme activity varied considerably within each group of animals and it was necessary to run separate controls each time the experiment was repeated in order to obtain a reliable baseline of activity.

Actinomycin D and cycloheximide increased tyrosine-α-ketoglutarate transaminase activity above the level obtained by force-feeding alone. This trend was observed in the animals pre-fed with glucose and also in the animals which were pre-fed with fructose (table 5). In addition, the effect of 8-azaguanine was also tested, but only in the animals pre-fed with fructose. This antibiotic did not induce tyrosine-α-ketoglutarate transaminase when given alone, nor did it prevent the induction produced by actinomycin D or cycloheximide (table 5).

It is significant that tyrosine-α-ketoglutarate transaminase was induced by actinomycin D or cycloheximide while the induction of glucose 6-phosphatase was blocked partially or totally; the induction of the transaminase, therefore, can not be ascribed to a mere irritation of the animals. Also, both antibiotics produced the expected results with respect to glucose 6-phosphatase activity, indicating that these antibiotics were acting as inhibitors of messenger RNA synthesis (actinomycin D) and protein synthesis (cycloheximide). Yet 8-azaguanine, which inhibited the induction of glucose 6-phosphatase after force-feeding casein hydrolysate, failed to induce tyrosine-α-ketoglutarate transaminase activity or to inhibit the superinduction of the transaminase by the other two antibiotics.

Finally, tyrosine-α-ketoglutarate transaminase was not induced substantially by either actinomycin D or cycloheximide in the animals force-fed with carbohydrate (table 6). A previous report indicates that

the induction of tyrosine-α-ketoglutarate transaminase by glucocorticoids can be greatly reduced by force-feeding carbohydrate (21). This probably accounts for the failure of the antibiotics to induce tyrosine-α-ketoglutarate transaminase in these experiments. The possibility that tyrosine-α-ketoglutarate transaminase induction was blocked by a lack of dietary protein or amino acids can be ruled out, because pyruvate kinase was induced, indicating that sufficient amounts of amino acids were present in the liver to allow for increased protein synthesis. Also, since the rise in pyruvate kinase activity after force-feeding carbohydrate was blocked by the antibiotics, this indicates that both actinomycin D and cycloheximide were retained by the body at least long enough to cause an inhibition of enzyme synthesis.

DISCUSSION

The induction of glucose 6-phosphatase by fructose, or a high protein regimen is believed to be at least partly independent of the release of cortisol (31), and associated with increased gluconeogenesis. The induction of this enzyme by a dietary stimulus then may be an indication of increased gluconeogenesis.

It is much more difficult, however, to explain why pyruvate kinase activity should be increased, even temporarily, after feeding a high protein regimen to animals pre-fed with a high carbohydrate, protein-free diet, when the demand under gluconeogenic conditions is for an increase rater than a decrease in phosphoenolpyruvate production. It is possible that a protein-free diet containing glucose can initiate a mechanism to conserve amino acids by reducing protein synthesis even if messenger RNA production is continued or increased. Refeeding protein to such animals may increase enzyme activity in general by relieving the inhibition of protein synthesis. Such regulation of protein synthesis may be advantageous for conserving nitrogen and amino acids, but may be disadvantageous by partially reversing gluconeogenesis. However, liver protein is maintained at a high level by dietary fructose, despite the absence of protein in the diet. It appears, then, that in vivo maintenance of homeostasis, may involve other factors in addition to an increase or decrease in the concentration of enzymes.

Another important point concerning the induction of glucose 6-phosphatase and pyruvate kinase is that when these inductions are inhibited by antibiotics a baseline of enzyme activity is still maintained. This suggests that glucose 6-phosphatase and pyruvate kinase both have an "enzyme reserve" which is relatively inert to dietary or other stimuli and another enzyme pool which is affected much more readily. For example, it has been shown that 2 isozymes of pyruvate kinase (one subject to dietary variation and another which is not) are present in the liver (32).

The increase in tyrosine-α-ketoglutarate transaminase activity caused by actinomycin D and cycloheximide to levels above and beyond those observed after force-feeding casein hydrolysate is very difficult to explain. Part of the difficulty is that this superinduction occurs while the induction of glucose 6-phosphatase is inhibited by both antibiotics. That is, if tyrosine-α-ketoglutarate transaminase activity were increased by actinomycin D only, this could be easily explained by assuming that the production of a messenger RNA specific for the repressor of tyrosine-α-ketoglutarate transaminase synthesis is inhibited, the repressor is not produced and transaminase activity, therefore, rises. But tyrosine-α-ketoglutarate transaminase is also induced by cycloheximide, which inhibits the induction of glucose 6-phosphatase to an even greater extent than actinomycin D. Since cycloheximide is thought to inhibit protein synthesis at the translation level, it would be expected to inhibit the synthesis of tyrosine-α-ketoglutarate transaminase rather than increase it.

There is a possibility that the translational step of protein synthesis is affected by antibiotics to a different extent for each enzyme, but there is no direct proof of this. And actinomycin D and cycloheximide induce tyrosine-α-ketoglutarate transaminase while inhibiting the induction of glucose 6-phosphatase and pyruvate kinase which raises the possibility that tyrosine-α-ketoglutarate transaminase induction with

these antibiotics is a result of diverting some portion of the amino acid pool of the liver from the synthesis of some enzymes to the synthesis of tyrosine-α-ketoglutarate transaminase. The inhibitory effect of actinomycin D on messenger RNA synthesis would not interfere with the induction of tyrosine-α-ketoglutarate transaminase if the messenger RNA specific for tyrosine-α-ketoglutarate transaminase was relatively long-lived. That cycloheximide also produces this induction, while inhibiting the induction of other enzymes, may be explained by assuming that cycloheximide at low concentrations does not prevent incorporation of amino acids into tyrosine-α-ketoglutarate transaminase, but does prevent tyrosine-α-ketoglutarate transaminase synthesis at higher doses (30). In these experiments the effect of such high doses over a 24-hour period is not known, because the animals injected even with 1 mg of cycloheximide died within 12 hours after injection.

An alternative possibility is that cycloheximide interferes with polysome formation. This again could lead to an increase in the synthesis of tyrosine-α-ketoglutarate transaminase by diverting the amino acid pool of liver to the synthesis of this enzyme. If this latter mechanism of tyrosine-α-ketoglutarate transaminase induction by antibiotics is correct, it would mean that tyrosine-α-ketoglutarate transaminase synthesis can be increased without increased polysome formation or that polysomes specific for tyrosine-α-ketoglutarate transaminase synthesis can be formed in the presence of antibiotics. In fact, some enzymes, such as serine dehydrase, require tryptophan in an amino acid mixture for induction (33); this suggests that the induction of serine dehydrase requires polysome formation, a process which is inhibited by the absence of tryptophan in the diet (34). This mechanism may not require the presence of a repressor as suggested by Garren et al. (26), though does not rule it out. The difficulty involved in ascertaining the mechanism of tyrosine-α-ketoglutarate transaminase induction is that the results seem to vary with the dose of actinomycin D given and also among investigators.

Another difficulty involves the diverse effects so far attributed to these antibiotics. For example, it has been reported that actinomycin D increases alkaline phosphatase activity in nine day old mice (35) and in the intestines of chick embryo (36), and cholesterol synthesis in rat liver (37). Also, actinomycin D reduces the absorption of amino acids in the intestine (38) and is believed to cause a defect in cardiac ribosomes (39). Yet it has been shown that actinomycin D reduces RNA and protein synthesis (39), especially the DNA-dependent synthesis of RNA (40, 41) by complexing with DNA and inhibiting RNA-polymerase (41). Cycloheximide inhibits protein synthesis in rat liver (42), in mammalian cell cultures (43), in cultured human lymphocytes (44), in yeast (45) and in a growing number of other tissues. But cycloheximide appears to have some side effects as well. For example, cycloheximide inhibits the synthesis of ribosomal RNA in cultured human lymphocytes (44), in yeast (46, 47) and *Neurospora crassa* (48), but not in mammalian cell cultures (43, 49). In addition, cycloheximide has been reported to stimulate incorporation of amino acids by isolated rat liver ribosomes (50) prepared for normal rats, but not from adrenalectomized rats.

It is necessary, therefore, to exercise caution in interpreting the results obtained by using actinomycin D and cycloheximide. This is necessary because it is possible that these antibiotics induce tyrosine-α-ketoglutarate transaminase by acting at an extra-hepatic site such as the adrenals, the pancreas, the pituitary or some other site.

Presently it may be said that the induction of tyrosine-α-ketoglutarate transaminase is affected differently by antibiotics than the induction of glucose 6-phosphatase and pyruvate kinase. The reasons for this are not clear and a great deal more work is required to ascertain the mechanism of induction of tyrosine-α-ketoglutarate transaminase as well as the secondary effects of antibiotics and the interaction of these with the regulatory systems of enzyme synthesis.

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Tissue Levels of Acetylcholine and Acetylcholinesterase in Weanling Germfree Rats Subjected to Acute Choline Deficiency [1]

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ABSTRACT Small intestine and kidney acetylcholine levels were assayed in choline-deficient and normal weanling Fischer germfree (GF) and open-animal-room (OAR) rats to investigate our thesis that choline deficiency results in decreases in kidney acetylcholine making the renal vasculature hyperreactive to vasopressor amines, resulting in vasospasm, ischemia, vascular rupture, and tubular necrosis. We previously found that OAR rats develop much more severe nephropathy than GF rats when each are fed choline-deficient diets, due principally, we believe, to differences in choline metabolism and energy production. Accordingly, we postulated that renal acetylcholine levels should drop faster in choline-deficient OAR rats than in GF rats. We found that 5 days of dietary choline deficiency (a time before there are signs of nephropathy histologically or by blood urea concentration) resulted in decreased concentrations of acetylcholine in the small intestine (32%) and kidneys (40%) of OAR rats. There was no fall in kidney acetylcholine levels in the GF rats whereas the small gut acetylcholine level decreased 24%. Small intestine acetylcholinesterase levels of GF and OAR rats were similar and not affected by choline deficiency. These data are consistent with the above stated thesis regarding the pathogenesis of the nephropathy of choline deficiency.

Hemorrhagic nephropathy is a major characteristic of acute choline deficiency of weanling rats (1–3). We postulated a vascular mechanism as central to the pathogenesis of the nephropathy based on the histologic findings and our finding a decrease in kidney acetylcholine levels of such rats. We believe the latter leads to an imbalance of vasoactive substances resulting in vasospasm and ischemia. We also reported that germfree rats placed on a choline-deficient diet developed much less renal injury as judged functionally and morphologically than open-animal-room rats (3). We therefore set out to determine if differences exist between the levels of acetylcholine in certain tissues of normal germfree and open-animal-room rats and how these levels changed in such rats subjected to dietary choline deficiency.

MATERIALS AND METHODS

The diet used for the production of acute choline deficiency is detailed elsewhere (3); the Salmon and Newberne (5) diet was modified by the addition of 5 g cholesterol and 0.4 g cystine/100 g diet, certain B vitamins, menadione, and inositol. The diet was steam autoclaved at 127° for 25 minutes.

Male rats of the Fischer strain,[5] 20–22 days old, were used for all experiments; housing, microbiologic testing, feeding regimens, handling, killing routine, tissue morphologic studies and acetylcholine and acetylcholinesterase analyses have been described elsewhere (3, 4). Acetylcholine levels were measured in the kidney and small intestine. The open-animal-room rats and the "germfree" [6] rats were divided

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[5] Purchased from the Charles River Breeding Laboratories, Wilmington, Massachusetts.

[6] The term "germfree" as used in this paper refers to rats free from viable bacteria, parasites, or fungi as determined by methods published elsewhere (9).

TABLE 1

Tissue levels of acetylcholine and acetylcholinesterase during choline deficiency

Tissue	Open-animal-room rats [1]						Germfree rats [2]					
	Acetylcholine			Cholinesterase			Acetylcholine			Cholinesterase		
	Choline [3]	Water		Choline	Water		Choline	Water		Choline	Water	
	(8) [4]	(8)		(8)	(8)		(7)	(7)		(7)	(7)	
	μg/g	μg/g	P	μg/hr/g [5]	μg/hr/g [5]	P	μg/g	μg/g	P	μg/hr/g	μg/hr/g	P
Small intestine	0.79 ± 0.04 [6]	0.54 ± 0.03	< 0.02	232 ± 23	219 ± 12	ns [7]	0.82 ± 0.03	0.63 ± 0.04	< 0.02	195 ± 13	190 ± 9	ns
Kidney	0.26 ± 0.02	0.16 ± 0.03	< 0.02	—	—	—	0.24 ± 0.02	0.27 ± 0.02	ns	—	—	—

[1] Open-animal-room, male rats of the Fischer strain, 20–22 days old at the start of the diet; rats were killed 5 days later.
[2] Germfree male rats of the Fischer strain, 20–22 days old at the start of the diet; rats were killed 5 days later.
[3] Choline chloride, 1.5 mg/ml in drinking water.
[4] Numbers in parentheses = numbers of rats. The experiment was conducted with 24 open-animal-room rats and 20 germfree rats; 4 of the 12 choline-deficient and 4 of the 12 choline-supplemented open-animal-room rats, and 3 of the 10 choline-deficient and 3 of the 10 choline-supplemented germfree rats were killed for histological examination of the kidneys. The remaining rats were killed for the measurements of acetylcholine and acetylcholinesterase in the tissues.
[5] Acetylcholine, μg, hydrolyzed per hour per gram tissue.
[6] Mean ± SE.
[7] ns = not significant.

each into 2 groups (by weight and litter) on the day of arrival, the day after weaning. They ate the choline-deficient diet ad libitum. Half the animals were given tap water to drink while the other half were given tap water containing 1.5 mg/ml choline chloride. The rats were weighed on arrival, 2 days later, and when killed on the morning of day 5.

Statistical comparisons of the data were made using the Student *t* test.

RESULTS

The kidney acetylcholine levels of the choline-supplemented germfree and open-animal-room rats were similar. When they were on choline-deficient diets for 5 days, the level of acetylcholine in the kidneys of the open-animal-room rats fell significantly (40%), but not at all in the germfree rats (table 1). The body weights, gross appearance and weights of the kidneys, blood urea nitrogen concentrations, hematocrits, and plasma total solid concentrations were similar in all rats. Microscopic examination of the kidneys of 3 of the 10 germfree choline-deficient and 4 of the 12 open-animal-room choline-deficient rats selected at random for examination showed no abnormalities (table 2).

The levels of small intestine acetylcholine of open-animal-room and germfree choline-supplemented rats were similar. In both germfree and open-animal-room rats fed the choline-deficient diet, the small intestine acetylcholine levels fell, 32% in open-animal-room rats and 24% in the germfree rats (table 1).

The levels of small intestine acetylcholinesterase were similar in all rats, choline supplemented or not.

DISCUSSION

Although germfree rats are substantially more resistant than open-animal-room rats to acute dietary choline deficiency, they are not completely resistant to choline deficiency as indicated by the increase in liver fat in germfree choline-deficient rats (3) and the decrease in intestinal acetylcholine levels which we have now observed. The small intestine acetylcholine concentrations are similar in both germfree and open-animal-room rats, both choline supplemented and choline defi-

TABLE 2

Body weight, kidney weight and blood urea nitrogen, hematocrit, and plasma total solids of open-animal-room (OAR) and germfree (GF) choline-deficient rats [1,4]

Status	Body wt		Kidney wt	Blood urea nitrogen	Hematocrit	Plasma total solids
	Start	End				
	g	*g*	*g*	*mg/100 ml*	*%*	*g/100 g*
OAR water (8) [2]	32±1	38±1	0.50±0.01	23±2	43±1	6.3±0.4
OAR choline (8) [3]	32±1	40±2	0.52±0.02	21±1	43±1	6.7±0.4
GF water (7)	27±1	35±1	0.50±0.02	27±12	40±1	6.3±0.4
GF choline (7)	27±1	38±1	0.54±0.01	15±1	39±1	6.9±0.3

[1] Open-animal-room (OAR) and germfree (GF) male rats of the Fischer strain, 20–22 days old at the start of the experiment; rats were killed after 5 days.
[2] Numbers in parentheses = numbers of rats.
[3] Choline chloride, 1.5 mg/ml drinking water.
[4] The data are presented as the mean ± SE. None of the differences in body weight, kidney weight, BUN, hematocrit or total solids were statistically significant.

cient, and the lower levels in the latter groups are not dependent on changes in acetylcholinesterase. We have no explanation for the lower levels of small intestine acetylcholine of the open-animal-room rats in our present experiments as compared with our previous experiments (4).

We postulated in a previous publication (4) that the nephropathy of acute choline deficiency was mediated via a decrease in kidney acetylcholine resulting in an imbalance of vasoactive neurohumoral substances, leading to renal ischemia, tubular necrosis and hemorrhage. This view is supported by observations of Nagler, Levenson and Baez [7] of the circulation of the mesoappendix of choline-deficient rats. We further theorized that one of the reasons that germfree rats develop less nephropathy than open-animal-room or conventionalized [8] rats under the conditions of nutritional choline deficiency might be due to differences in their responsiveness to vasoactive materials. In this regard, Baez and Gordon (6) found the microcirculation of the mesoappendix of germfree rats hyporesponsive to epinephrine when compared with that of open-animal-room rats. In addition, as explanations for dramatically less nephropathy in the germfree rats fed choline-deficient diets we had offered the following: (a) absent conversion of choline to trimethylamine in the germfree, a reaction which is brought about in the rat solely by intestinal bacteria (7), (b) absent utilization of methionine by intestinal bacteria in the germfree,[9] (c) lower metabolic rate in the germfree (8), and (d) possibly increased endogenous hepatic synthesis of choline by germfree rats (since demonstrated by Kwong, Fiala, Barnes, Kan and Levenson [10] for germfree rats on choline-deficient but not on choline-supplemented diets). All of these diminish the requirement of germfree rats for dietary choline.

In our present experiments, we have shown that the rapid decrease in kidney acetylcholine, which is characteristically found in the open-animal-room weanling rat after 5 days on the choline-deficient diet and which we believe to be fundamental to the development of the acute choline deficiency nephropathy, does not occur in germfree rats at this time. This is consistent with our view that the nephropathy is mediated via a neurohumoral vascular mechanism.

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Aspects of Lipid Metabolism in Ethanol-induced Fatty Liver

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ABSTRACT The effects of ingestion by the rat of a single large dose of ethanol on liver lipid composition, on lipogenesis in vitro, on serum lipoproteins, and on liver content of ATP and biotin were studied. The effect of ATP and adenine administration in those rats given ethanol was also investigated. Furthermore, the effect of ethanol in biotin-deficient rats was studied. In the liver of ethanol-treated rats neutral fat increased, while the incorporation in vitro of acetate-1-^{14}C into total lipids and the liver content of ATP decreased, when compared with the control rats receiving glucose in isocaloric amounts. No significant difference was observed in serum α- and β-lipoprotein levels and in liver biotin. The administration of 150 mg of ATP in ethanol-treated rats did not prevent fatty liver, whereas the lipogenesis and cellular levels of ATP were normalized. The administration of adenine to ethanol-treated rats caused a significant decrease of lipid content as compared with rats receiving only ethanol. Lastly, it was found that the administration of ethanol also in biotin-deficient rats caused the development of liver steatosis. The relationships between ATP and ethanol fatty liver are discussed in relation to possible mechanisms involved in the development of steatosis.

Ethanol ingestion by the rat is known to induce a fatty infiltration in the liver. The triglycerides that accumulate in the liver appear to be derived in large part from peripheral fat depots (1). In addition, either increased hepatic lipogenesis (2) or decreased fatty acid oxidation (3), might contribute to the increase of liver triglycerides.

In the present paper the effects of a single large dose of ethanol on lipid composition of rat liver, on incorporation of acetate-1-^{14}C into liver lipids in vitro, on serum lipoprotein levels, and on liver content of ATP and of protein-bound biotin are reported. The effects of the administration of ATP or adenine together with ethanol were also investigated. In fact, it had been previously observed that ethanol-induced fatty liver might be associated with a decrease of liver ATP and that injections of ATP may prevent fat accumulation (4). Furthermore, the effect of ethanol on lipid composition and on lipogenesis in the liver of biotin-deficient rats was studied since relationships between biotin and fatty liver were again noted in those animals treated with orotic acid (5), or with ethionine (6).

EXPERIMENTAL

Female weanling albino rats of the Wistar strain, weighing 40–45 g, were housed in cages with wire bottoms and fed ad libitum a basal diet. The composition of the diet was: (in percent) vitamin-free casein, 20; sucrose, 59; autoclaved egg white, 11; groundnut oil, 5; salt mixture IV XX, 4; and vitamin mixture XX, 1. After 60 days the animals were divided into 5 groups and subjected to an 8-hour fast. The rats in group 1 were given glucose 65.5% (w/v) by stomach tube, 2 ml/100 g body wt (equal isocalorically with the dose of ethanol). Rats of group 2 were treated with ethanol 47%, 2 ml (equal 0.75 g) per 100 g body wt; rats in groups 3 and 4 received the same dose of ethanol plus 1 ml neutralized aqueous solution of ATP, subcutaneously, containing 50 mg/ml or 100 mg/ml, respectively, 3 times during the experiment. The rats in group 5 received the same dose of ethanol plus 1 ml neutralized aqueous solution of adenine sulfate (40 mg/ml), subcutaneously, 3 times during the experiment. Sixteen hours after the ethanol treatment venous blood from the rats in

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the 5 groups was collected. Then the rats were killed and the livers saved for the determinations referred to above.

Blood was allowed to clot at 37° for 30 minutes and serum was separated after centrifuging at 3000 rpm for 15 minutes. Paper electrophoresis of serum proteins was carried out in veronal buffer, pH 8.6 and *I* 0.1 (7).

Total lipids were measured gravimetrically after extraction by a method described previously (8); cholesterol was estimated by the method of Sperry and Webb (9) and phospholipids by phosphorous analysis in total lipids (10).

Protein-bound biotin was assayed microbiologically with *Lactobacillus arabinosus* 17/5 ATCC 8014 on samples of liver homogenate autoclaved with 6 N H_2SO_4 at 121° for 60 minutes.

To determine the hepatic content of ATP the livers were quickly removed, frozen at − 70° (acetone-solid CO_2 mixture), homogenized with 4 volumes of perchloric acid, and centrifuged at 5,000 × *g* for 15 minutes. The supernatants were neutralized to pH 6.5 with 0.5 N KOH and the precipitated $KClO_4$ was removed. Aliquots of extracts were placed in 1 cm glass cuvettes with 250 μmoles of triethanolamine buffer, pH 7.6, 10 μmoles of $MgSO_4$, 20 μmoles of 3-phosphoglycerate, and 0.6 μmole of NADH. The decrease in optical density at 340 mμ was measured 8 minutes after the addition to the reaction mixture of a 0.03 ml suspension containing glyceraldehyde-3-phosphate dehydrogenase (4 mg protein/ml) and phosphoglycerate kinase (1 mg protein/ml).

Liver slices (500 to 600 mg) were prepared with a Stadie-Riggs slicer and incubated in 25 ml Erlenmeyer flasks containing 5 ml of calcium-free Krebs-Ringer phosphate medium, pH 7.4, 5 μmoles of α-ketoglutarate, and 0.5 μmole of sodium acetate-1-^{14}C [1] (specific activity 10.5 mCi/mmole). The flasks were shaken at 37° for 1 hour in air and the reaction was stopped by placing the flasks on ice. After removal of the medium the slices were rinsed 3 times with ice-cold buffer and homogenized in 20 volumes of methanol-chloroform (3:1 v/v). The extract was

[1] Obtained from the Radiochemical Center, Amersham, Bucks, England.

TABLE 1

Effect of acute ethanol ingestion and ATP or adenine administration on the composition of liver lipids and on the incorporation of acetate-1-^{14}C in vitro into liver lipids of rats

Group [1]	Animals in experiment	Liver lipids				Incorporation of acetate-1-^{14}C into liver lipids
		Cholesterol	Phospholipids	Neutral	Total	
		mg/g wet tissue				*cpm/g wet tissue*
1	Glucose-treated	2.85 ± 0.38 [2]	35.1 ± 1.48	20.2 ± 1.30	58.2 ± 1.32	24,557 ± 1,211
2	Ethanol-treated	3.02 ± 0.07	35.5 ± 1.33	79.8 ± 7.50	118.3 ± 7.32	14,578 ± 1,369
3	Ethanol-treated plus ATP, 150 mg	3.34 ± 0.09	37.3 ± 1.17	81.9 ± 7.98	122.5 ± 4.37	21,796 ± 3,080
4	Ethanol-treated plus ATP, 300 mg	2.91 ± 0.60	36.2 ± 2.98	62.2 ± 6.12	101.3 ± 4.72	22,287 ± 1,822
5	Ethanol-treated plus adenine, 120 mg	3.21 ± 0.18	34.9 ± 2.16	49.2 ± 7.17	87.3 ± 8.25	25,296 ± 1,184

[1] Eight rats per group.
[2] Averages ± SE of mean.

TABLE 2

Effect of acute ethanol ingestion and ATP or adenine administration on the hepatic levels of protein-bound biotin and of ATP in rat liver

Group [1]	Animals in experiment	Liver protein-bound biotin	Liver ATP
		μg/g wet tissue	*mμmoles/g wet tissue*
1	Glucose-treated	1.40 ± 0.04 [2]	1,613 ± 92
2	Ethanol-treated	1.49 ± 0.08	948 ± 72
3	Ethanol-treated plus ATP, 150 mg	1.56 ± 0.09	1,561 ± 99
4	Ethanol-treated plus ATP, 300 mg	1.73 ± 0.12	2,035 ± 153
5	Ethanol-treated plus adenine, 120 mg	1.65 ± 0.11	2,074 ± 188

[1] Six rats per group.
[2] Averages ± SE of mean.

washed with 0.2 its volume of salt solution (0.02% $CaCl_2$, 0.017% $MgCl_2$, and 0.29% NaCl), and the resulting mixture separated into two phases. The lower phase, containing total lipid, was washed 3 times with a solvent mixture (chloroform, methanol and water (3:48:47 v/v)). The washed extract was evaporated to dryness and the residue was dissolved, first in petroleum ether (bp 40 to 60°) and then in chloroform. Samples of this solution were taken for radioactivity measurements in a windowless gas-flow counter.

In a second experiment to determine the effect of biotin deficiency on ethanol-induced fatty liver, female weanling albino rats of the Wistar strain were fed for 60 days on a biotin-deficient diet with the same composition as described above, but the autoclaved egg white was replaced by raw dried egg white and the vitamin supplement was biotin-free.

At the end of the test period the rats were divided into 2 groups. A group of deficient rats (group 6) was treated with glucose while another group (group 7) was treated with ethanol.

The animals were killed after 16 hours and the livers used for the determination of lipid composition and for the acetate-1-^{14}C incorporation in vitro into total lipids.

All data were analyzed by statistical procedures.

RESULTS

Paper electrophoresis of serum lipoproteins showed no significant differences of α- and β-lipoproteins levels in those rats treated with ethanol (group 1), or with ethanol plus ATP (groups 3 and 4), or with adenine (group 5), as compared with the rats receiving glucose in isocaloric amounts (group 2).

From table 1 it appears that total lipid content was significantly increased ($P < 0.001$) in ethanol-treated rats when compared with the glucose-treated ones. This increase was due entirely to the neutral lipid fraction, whereas no significant differences ($P < 0.001$) were found in phospholipid and cholesterol fractions.

A remarkable increase of fat content of liver also was observed in ethanol-treated rats and in those injected with 150 mg of ATP (group 3) ($P < 0.001$).

The fat content of livers of rats treated both with ethanol and 300 mg of ATP showed a slight but significant decrease ($P < 0.05$) as compared with ethanol-treated rats (group 2). A notable decrease ($P < 0.01$) was observed in rats receiving both ethanol and adenine (group 5).

The data, concerning the lipid synthesis in vitro, showed that the rate of incorporation of acetate-1-^{14}C into total lipids of liver slices from ethanol-treated rats was lower than that of rats of group 1 ($P < 0.001$).

No significant difference of acetate-1-^{14}C incorporation was noted between those animals treated either with ATP (groups 3 and 4) or adenine (group 5), and the control rats (group 1).

No significant changes in the liver content of protein-bound biotin were observed among glucose-treated rats, ethanol-treated rats, and ethanol-treated rats injected with ATP or adenine (table 2). The cellular content of ATP decreased in the liver of rats receiving ethanol when compared with control rats ($P < 0.001$); no differences were noted in the rats treated with both ethanol and ATP, or with ethanol and adenine.

TABLE 3

Effect of acute ethanol ingestion on the composition of liver lipids and on the incorporation of acetate-1-^{14}C in vitro into liver lipids of biotin-deficient rats

Group [1]	Animals in experiment	Liver lipids				Incorporation of acetate-1-^{14}C into liver lipids
		Cholesterol	Phospholipids	Neutral	Total	
		mg/g wet tissue				*cpm/g wet tissue*
6	Biotin-deficient, treated with glucose	3.12 ± 0.11 [2]	33.58 ± 1.26	19.47 ± 1.31	57.17 ± 2.16	15,080 ± 1,109
7	Biotin-deficient, treated with ethanol	2.88 ± 0.09	36.54 ± 1.66	56.52 ± 5.99	95.94 ± 6.07	7,095 ± 842

[1] Eight rats per group.
[2] Averages ± SE of mean.

It appears from the data in table 3 that the acute ethanol ingestion also caused a marked increase of liver lipid content in biotin-deficient rats (group 6) when compared with the biotin-deficient rats which did not receive ethanol (group 7) ($P < 0.001$). The increase was due entirely to neutral fat. The incorporation of acetate-1-^{14}C into liver total lipids of biotin-deficient rats was significantly decreased ($P < 0.001$) by acute ethanol ingestion.

DISCUSSION

The neutral fat increase in the liver of ethanol-treated animals has been ascribed either to increased fatty acid synthesis (2), or to decreased fat secretion from the liver (11), or to increased fatty acid mobilization from peripheral fat depots to the liver (1). According to the data of Horning et al. (12) and of Scheig and Isselbacher (1), our preliminary experiments showed that the most probable cause of fatty liver formation seemed to be the stimulation of fatty acid mobilization from extrahepatic stores to the liver (13). In fact, the percentage fatty acid composition of total liver lipids in ethanol-treated rats strongly resembles that of adipose tissue. Moreover, the actual amount of linoleate of the liver was increased, which could indicate that mobilization from peripheral fat depots had occurred.

The present data, however, appear to exclude the hypothesis that the development of ethanol fatty liver may result from an increase of lipogenesis. In fact, the incorporation of acetate-1-^{14}C into total lipids of liver slices from ethanol-treated rats was decreased. Moreover, in biotin-deficient rats ethanol ingestion produced a fatty liver although a notable decrease in the content of biotin enzymes, and in particular of acetyl-CoA-carboxylase, was observed (14). Actually it has been suggested that the development of some types of liver infiltration (i.e., orotic acid-fatty liver), which are associated with a stimulation of lipid synthesis (15) is inhibited by biotin deficiency (5).

Also, decreased conversion of triglycerides to phospholipids does not appear to have any association with the development of the steatosis. In fact, the concentration of phospholipids in the liver was

unchanged by ethanol treatment. Moreover, the addition of cytidine diphosphate (CDP) to the homogenates from ethanol-treated rats stimulated phospholipid formation (1), suggesting that the phospholipid synthesizing system was reasonably intact. Furthermore, there is no evidence that the ethanol-induced fatty liver is determined by impaired secretion of lipids from liver, since the concentration of serum lipoproteins was unchanged by ethanol treatment, as also observed by Seakins and Robinson (16). It has been suggested that this type of fatty liver might result from impaired secretion of lipids from the liver as a consequence of decreased lipoprotein synthesis, induced by acute ATP deficiency (4, 11). Actually the relationships between ethanol-induced fatty liver and ATP have not yet been completely elucidated. Ethanol ingestion produced a decrease of liver ATP. The administration of 150 mg of ATP, however, normalized the hepatic content of ATP but did not prevent the development of fatty liver. Only the administration of adenine, or to a lesser extent of 300 mg of ATP, partially prevented the steatosis.

From these data it appears that the mechanisms of protection afforded only by very large doses of ATP or adenine still remain uncertain. The possibility cannot be excluded that the protective action of ATP may be mediated through pharmacological mechanisms (shock, hypothermia) rather than through biochemical mechanisms (4, 17).

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Nickel Toxicity in Growing Chicks [1]

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ABSTRACT Two experiments have been conducted with chicks to determine the effects of high levels of dietary nickel on growth and nutrient utilization. Dietary nickel was supplied as either the acetate or sulfate salt at levels up to 1300 ppm in a basal diet calculated to be adequate in all known nutrients. Growth of chicks to 4 weeks of age was significantly depressed at 700 ppm nickel and above.

Metabolizable energy determinations on the respective experimental diets suggested an impairment in energy metabolism at the higher levels of dietary nickel. Fat retentions were not affected by nickel, but a marked reduction in nitrogen retention was obtained with the higher dietary levels of nickel.

In a second experiment, 1100 ppm nickel were incorporated into a basal diet and control diets pair-fed to delineate the effects associated with feed consumption and nickel toxicity per se. No significant differences in growth rate were obtained with either 1100 ppm nickel as the sulfate or acetate in comparison with the pair-fed controls. Nitrogen retention values were depressed in birds fed 1100 ppm nickel.

The effects of nickel have been clearly defined in rats, guinea pigs and mice. In a study with rats, it was found that levels of 250, 500 and 1000 ppm of nickel in three different forms did not significantly affect growth rate or reproduction (1). Approximately 71 to 91% of ingested Ni was found in the feces. Appreciable quantities were also retained in the tissues. In a further study, young rats fed on a diet containing 250 ppm of added Ni for 16 months grew normally (2). They found that maximum Ni levels occurred after 8 months and gradually decreased thereafter, probably because of decreased absorption of the nickel together with continued excretion. Low molar concentrations of nickel given to white mice by means of intraperitoneal injection were found to be lethal (3). Nickel given to guinea pigs by subcutaneous injections over a period of 120 days showed that the nickel was present in all organs investigated, and was eliminated primarily by the kidneys (4). Studies on the effect of Ni on various physiological systems have shown that Ni does activate arginase (5), carboxylase (6) and trypsin (7). Acid phosphatase under certain conditions was inhibited (8).

In view of the lack of information relating to the toxicity of nickel in poultry and the difficulties of extrapolating data from studies with rats and mice to chickens, the present study was initiated to evaluate the effects of high levels of this element on growth and the utilization of protein, fat and energy in the chick.

EXPERIMENTAL

Two studies were carried out with Hubbard broiler chicks grown to 4 weeks of age in batteries with raised wire floors. In the first experiment, nickel sulfate or nickel acetate was fed in amounts to supply zero, 100, 300, 500, 700, 900, 1100 and 1300 ppm added nickel to the basal diet. These experimental diets were supplied ad libitum.

In a second study, the 1100 ppm level of nickel was fed as the sulfate or acetate, and the basal diet was pair-fed with these diets to evaluate the separate effects of nickel on feed consumption and protein utilization. An additional treatment employed the basal diet fed ad libitum.

In each of the experiments, three replicate groups of 8 chicks each (4 males and 4 females) were fed each of the experimental diets. The basal diet (table 1) used in these studies was calculated to be adequate in all essential nutrients.

In addition to body weight gain and feed consumption data, feces samples

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TABLE 1

Diet of nickel sulfate and acetate used in chick growth study

Ingredient	% of diet
Fish meal	5.00
Alfalfa meal (17% protein)	2.00
Whey, dried	1.00
Corn, diet, dried sol.	1.00
Animal fat	5.00
Ground yellow corn	49.50
Soybean meal (44% protein)	31.15
Dicalcium phosphate	1.00
Calcium carbonate	0.75
Salt	0.20
Manganese dioxide	0.02
DL-Methionine	0.10
Vitamin mix [1]	2.50
Chromium oxide	0.20
Total	100.00

[1] Supplied the following per kilogram of diet: (in mg) ascorbic acid, 12.5; thiamine·HCl, 12.5; niacin, 100.0; riboflavin, 20.0; pyridoxine·HCl, 12.5; *d*-biotin, 1.25; Ca D-pantothenate, 75.0; vitamin B_{12} (0.1%), 10.0; folic acid, 4.00; *d*-α-tocopheryl acetate, 200.0; menadione (2-methyl-naphthoquinone), 1.25; ethoxyquin, 500.0; *i*-inositol, 500.0; *p*-aminobenzoic acid, 25.0; oxytetracycline, 25.0; (in IU) vitamin A palmitate (stabilized), 14,000; and vitamin D_3, 1,500.

were collected during the fourth week of each experiment to determine the effects of the nickel on nitrogen, calcium, phosphorus and fat retentions and on dietary metabolizable energy values.

A 0.20% level of chromium oxide (Cr_2O_3) was mixed into the experimental rations as an inert marker and the ratio of this material to the analysis values obtained for the nutrients listed above was used to calculate retentions according to the formula:

$$\% \text{ retention of "X"} = 100 - 100 \frac{(Cr_2O_3 \text{ feed}}{(Cr_2O_3 \text{ feces}} \times \frac{\text{"X" feces)}}{\text{"X" feed)}}$$

Chromium oxide was determined on feed and feces samples by $HNO_3 - HClO_4$ digestion as outlined by Edwards and Gillis (9). The perchloric acid digest from the above determination was used for the determination of calcium by flame photometry employing a phosphorus correction (10) and for the determination of total phosphorus (11). The A.O.A.C. Kjeldahl method was used for nitrogen determinations while combustible energy values were determined with the Parr oxygen bomb calorimeter.

All data, where applicable, were subjected to statistical analysis by analysis of variance and the means separated by Duncan's multiple range test (12).

RESULTS AND DISCUSSION

No significant differences were obtained in the growth of chicks fed the two forms of nickel (tables 2 and 3). The birds fed nickel sulfate showed no significant differences in body weights up to the 300 ppm level, but the feeding of 500 to 700 ppm Ni significantly depressed weight gains in comparison with the control birds (table 2). A further progressive growth reduction occurred at the 900 to 1300 ppm levels of nickel. Feed conversion was not altered up to the 900 ppm level, at which point it was increased with each increasing level of nickel.

Nickel acetate gave results similar to those of nickel sulfate (table 3). No significant differences in body weights occurred up to the 500 ppm level but body weights were reduced at the 900 ppm level of nickel. Thus, nickel caused a progressive growth depression when fed as either the acetate or sulfate salts. Feed conversions were apparently not affected up to the 900 ppm nickel level but were depressed at both the 1300 ppm levels. Calculated amounts of nickel consumed per bird were quite similar for the two sources (tables 2 and 3). The 500 to 700 ppm levels appeared to be in a plateau region of intake. These results suggested that the amount of nickel ingested controlled the level of feed consumption. The amount of nickel ingested per bird, calculated as milligrams consumed per gram of gain, gave opposing data (tables 2 and 3). These data showed that as the birds ingested increasing amounts of nickel, body weight decreased in a direct relationship and that the effect of nickel was in addition to reduced feed intake. This problem was further examined in studying the metabolism of feed nutrients.

The effects of nickel on the utilization of dietary nutrients was investigated by means of Cr_2O_3 marker techniques. No great differences were found in metabolizable energy values or percent fat absorption with birds fed nickel sulfate (table 4). Metabolizable energy values were unal-

TABLE 2

Effect of dietary nickel sulfate on body weights, feed utilization and levels of nickel ingested in chicks

Nickel added as nickel sulfate	Body wt, 4 weeks	Feed conversion	Calculated nickel ingested to gain	Calculated nickel consumed per bird
ppm	*g*		*mg/g*	*mg*
0	565 [a 1]	1.78	T [2]	T
100	534 [a]	1.73	151	87
300	568 [a]	1.68	453	269
500	467 [ab]	1.69	687	406
700	376 [b]	1.97	794	412
900	247 [c]	2.11	837	396
1100	180 [c]	2.38	889	373
1300	179 [c]	2.82	1,347	478

[1] Means having different superscripts are statistically different at the 0.05 level of probability.
[2] T = trace.

TABLE 3

Effects of dietary nickel acetate on body weights, feed utilization and levels of nickel ingested in chicks

Nickel added as nickel acetate	Body wt, 4 weeks	Feed conversion	Calculated nickel ingested to gain	Calculated nickel consumed per bird
ppm	*g*		*mg/g*	*mg*
0	565 [a 1]	1.78	T [2]	T
100	514 [a]	1.79	152	85
300	559 [a]	1.66	429	259
500	484 [ab]	1.71	656	383
700	390 [b]	1.79	795	444
900	259 [c]	2.13	870	409
1100	256 [c]	2.04	1009	483
1300	173 [c]	2.54	1155	454

[1] Means having different superscripts are statistically different at the 0.05 level of probability.
[2] T = trace.

TABLE 4

Effect of nickel sulfate on the metabolism of some dietary nutrients

Nickel added	Gross energy retention	Metabolizable energy	Fat retention	Nitrogen retained	Protein efficiency ratio
ppm	*%*	*kcal/g feed*	*%*	*%*	
0	64.83	2.72	69.46	41.16	2.44
100	63.96	2.68	67.06	41.64	2.51
300	65.66	2.76	69.58	41.58	2.59
500	62.29	2.61	64.80	33.00	2.57
700	56.37	2.37	68.33	19.57	2.21
900	58.03	2.44	72.50	16.17	2.06
1100	56.06	2.35	75.75	12.50	1.83
1300	52.05	2.19	71.79	11.27	1.54

tered up to the 500 ppm level of nickel sulfate but appeared to be decreasing at levels above 500 ppm. Percent nitrogen retentions showed a decrease between the 300 to 500 ppm nickel levels. Nitrogen retentions dropped from 41% at the 300 ppm level to 33% at the 500 ppm level. It was further observed that as the level of nickel increased, the amount of nitrogen retained decreased. Protein efficiency ratios were found to decrease with higher levels of nickel sulfate. Above the 500

ppm level of the diet a decrease occurred in the PER with each increasing amount of nickel fed above this level.

Nickel acetate, when compared with nickel sulfate, showed no significant alterations in either the gross energy or fat retained (table 5). The metabolizable energy was unaffected by nickel acetate up to the 1100 ppm Ni level. The feeding of 1300 ppm Ni did not further lower the metabolizable energy figures. Percent nitrogen retained was decreased by nickel acetate feeding but no differences occurred until a 900 ppm Ni level was fed, and for each increment added thereafter, a further reduction occurred. The PER values of nickel acetate were found to be slightly different from those of nickel sulfate in that a decrease did not occur until the 900 ppm Ni level was fed. A further reduction in PER values occurred with higher levels of nickel acetate (table 5).

These results do not agree with the in vitro work of Sugai (7) who found an increase in trypsin activity with nickel. Our experimental data would suggest a reduction of proteolytic enzyme activity or increased protein catabolism. The question of whether the availability of nickel from the two sources was a factor in the differences noted between the sulfate and acetate salts or whether the sulfate ions were responsible for the differences in results noted cannot be resolved at this point.

In view of the apparent effects of dietary nickel on both feed consumption and nitrogen utilization, an additional experiment involving the feeding of 1100 ppm nickel was conducted. In this study, control groups were pair-fed at a level equivalent to the feed consumption of birds on the nickel-containing diets. When feed intake was equalized, there was no signifi-

TABLE 5

Effect of nickel acetate on the metabolism of some dietary nutrients

Nickel added	Gross energy retention	Metabolizable energy	Fat retention	Nitrogen retained	Protein efficiency ratio
ppm	%	*kcal/g feed*	%	%	
0	64.83	2.72	69.46	41.16	2.44
100	63.43	2.66	69.78	33.96	2.43
300	63.89	2.68	64.97	36.99	2.62
500	65.80	2.76	71.79	36.67	2.54
700	64.56	2.71	72.49	36.11	2.43
900	62.56	2.63	79.06	28.28	2.04
1100	58.33	2.45	73.03	20.34	2.08
1300	58.48	2.46	75.26	15.82	1.71

TABLE 6

Effect of dietary nickel on pair-fed chicks

Dietary treatment	Avg body wt 4 weeks	Feed consumed per bird	Feed conversion	Nitrogen retention
	g	*g*		%
1. Basal diet ad libitum	570 [a] [1]	905	1.74	54.4
2. 1100 ppm Ni as nickel acetate ad libitum	304 [b]	538	2.12	44.9
3. Basal diet pair-fed with treatment 2	292 [b]	501	2.07	58.1
4. 1100 ppm Ni as nickel sulfate ad libitum	262 [b]	490	2.31	46.5
5. Basal diet pair-fed with treatment 4	259 [b]	451	2.16	63.4

[1] Means having different superscripts are statistically different at the 0.05 level of probability.

cant effect of dietary nickel on the growth rate of chicks to 4 weeks of age (table 6). No significant differences were noted between the acetate and sulfate salts. Feed conversions were slightly better in the pair-fed groups not receiving nickel. Nitrogen retentions were decreased with the feeding of 1100 ppm nickel, as either the acetate or sulfate, in comparison with the pair-fed control groups (table 6).

These data, along with the results obtained in the previous study, suggest that nickel, in addition to having an effect on feed intake, was detrimental to nitrogen retention. A striking difference in nitrogen retention values was obtained with nickel supplementation between the two experiments. The birds in the second study showed much higher nitrogen retention values at 1100 ppm nickel than in the first experiment. These differences are explained.

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Influence of Ascorbic Acid on the Absorption of Copper by Rats

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ABSTRACT Experiments were conducted to determine the effects of high levels of ascorbic acid on rats and to determine which step or steps in the utilization of copper are influenced by these high levels of ascorbic acid. The effects of high levels of dietary ascorbic acid on rats are much less severe than those reported for chicks and rabbits. Ascorbic acid significantly depressed the absorption of ^{64}Cu when the acid was put into a ligated intestinal segment along with the radiocopper. A smaller, non-significant, depression in ^{64}Cu resulted when ascorbic acid was given intraperitoneally and ^{64}Cu was put into the ligated segment. In other experiments, the whole-body retention of a single dose of ^{64}Cu was determined. Rats fed a diet containing 1% ascorbic acid retained less of an orally administered dose of ^{64}Cu than did the controls; however, when ^{64}Cu was given intraperitoneally, retention was not significantly affected by 1% dietary ascorbic acid. Thus, the results of both ligated segment studies and whole-body retention experiments indicate that, in rats, ascorbic acid depresses the intestinal absorption of copper, but has little or no effect on copper excretion.

The copper status of an animal is influenced by a number of dietary factors other than the level of copper in the diet. These factors include zinc (1–8), cadmium (8, 9), mercury and silver (10), molybdenum and sulfate (11–13), and phytate (14). Recently, ascorbic acid was added to this list when it was found that its inclusion in the diets of chicks (15, 16) or rabbits (17) increased the severity of copper deficiency.

At least three possible explanations for the effect of ascorbic acid on copper deficiency are immediately evident: 1) interference with the absorption of copper from the intestine; 2) interference with transport and function of copper at the cellular level; or 3) increased copper excretion. The objectives of the study reported here were: 1) to determine if high dietary levels of ascorbic acid increase the severity of copper deficiency in rats as they do in chicks, and 2) to determine which step or steps in copper utilization are affected by ascorbic acid.

MATERIALS AND METHODS

Experiments with ligated intestinal segments. Male rats of the Sprague-Dawley strain that had been housed in stainless steel cages and fed a commercial diet [1] were used in these experiments. Rats weighing from 250 to 350 g were used and were assigned to replicates in a randomized block design according to body weight.

Radiocopper, received as $Cu(NO_3)_2$, was diluted to a copper concentration of 2 μg/ml with either distilled water or a 0.5% solution of ascorbic acid. Each rat received 1.0 μg of copper and either zero or 2.5 mg of ascorbic acid.

Rats were given ^{64}Cu either intraduodenally via a ligated loop technique (7, 18), or by intraperitoneal (IP) injection. Four treatment combinations were used with those rats that received ^{64}Cu via the ligated loop: 1) control (no ascorbic acid); 2) ^{64}Cu and ascorbic acid were both given intraduodenally; 3) ascorbic acid was given intraperitoneally at the same time the ^{64}Cu was administered intraduodenally; and 4) ascorbic acid was given intraperitoneally 2 hours before intraduodenal administration of ^{64}Cu. In addition to these four treatments in which the ^{64}Cu was given intraduodenally, two treatments involving intraperitoneal administration of

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[1] Big Red Dog Chow, Agway, Inc., Syracuse, New York. Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

^{64}Cu were included: 1) control (no ascorbic acid); and 2) ^{64}Cu and ascorbic acid were given intraperitoneally simultaneously.

Three hours after administration of ^{64}Cu, a blood sample was taken, the rats were anesthetized with ether and decapitated. The ^{64}Cu concentrations in the blood, heart, kidneys, and liver were determined and the ligated segment and its contents were separated and counted. All counting was done in a well-type scintillation counter. The results of the ligated loop studies were subjected to an analysis of variance and means were compared using a multiple range test (19). Statements of significance are based on odds of at least 19 to 1.

Purified diet experiments. Male rats of the Sprague-Dawley strain also were used in these experiments. In trials 1, 2, and 3, mated female rats were maintained on the stock diet until just before parturition. At that time, they were put into individual cages and had access to both the stock diet and a copper-deficient purified diet (table 1). When the litters were 2 weeks old, the stock diet was removed and only the purified diet and distilled water were available. In trial 4, essentially the same procedure was used, except that the purified diet was introduced at the same time that the male rats were put in with the females for breeding. The male rats were left for 8 days and, during this period, both the stock diet and the purified diet were available. When the males were taken out, the stock diet was also removed. From then until weaning, only the copper-deficient diet and distilled water were available.

TABLE 1

Composition of the copper-deficient diet

	%
Dried skim milk	60.0
Sucrose	27.3
Corn oil	10.0
Methionine	0.5
Choline chloride	0.15
Vitamins [1]	1.0
Minerals [2]	1.0

[1] Supplies: (per 100 g of diet) Vitamin A, 500 IU; vitamin D_3, 98 ICU; α-tocopherol, 60 mg; vitamin K (klotogen F), 0.15 mg; thiamine, 1 mg; riboflavin, 1 mg; pyridoxine·HCl, 0.45 mg; niacin, 5 mg; Ca pantothenate, 2 mg; and B_{12}, 2 μg.

[2] Supplies: (per 100 g of diet) $FeSO_4 \cdot 7H_2O$, 12.5 mg; $MnSO_4 \cdot H_2O$, 15.4 mg; and KI, 50 μg.

Young male rats were weaned when 21 days old and were assigned to treatments in a randomized block design. Replications were on the basis of litters and body weight. The four diets that were used were: 1) the copper-deficient basal diet; 2) basal diet + 1% ascorbic acid; 3) a copper-adequate control diet, and; 4) control diet + 1% ascorbic acid. All diets were stored in a cold room at 2 to 4°. The rats were fed these diets for 9 weeks and were weighed at weekly intervals.

Survivors from trials 1 and 2 were sacrificed at the end of the experiment and liver copper concentrations were determined. Survivors from trials 3 and 4 were dosed with ^{64}Cu, and retention of both intraperitoneal and orally administered radiocopper was determined by whole-body counting. The procedure for rats from trials 3 and 4 was as follows: At the end of week 8 of the experiment, all survivors received a single intraperitoneal injection of 10 μg of copper labeled with ^{64}Cu and were subsequently counted periodically in the whole-body counter.[2] One week later, after this initial dose of ^{64}Cu (half-life = 12.8 hours) had decayed to background levels, the rats were given a second dose of ^{64}Cu by stomach tube. In all cases, rats were counted immediately after receiving radiocopper and were recounted at periodic intervals. The first count for each rat was arbitrarily set at 100%, and subsequent counts were expressed as a percentage of the initial count. Results of each count were subjected to an analysis of variance and individual means were compared by a multiple range test (19). Statements of significance are based on odds of at least 19 to 1.

The rats assigned to the control diets in trial 3 were inadvertently fed the wrong diet, therefore no data were obtained for copper-adequate rats from this trial. In trial 4, all the rats fed copper-deficient diets died during the first 5 weeks of the experiment, and in this trial, ^{64}Cu retention was measured only in copper-adequate rats.

[2] The whole-body counter consisted of a Nuclear-Chicago Tobor detector connected to a Nuclear-Chicago Model 8725 single-channel analyzer.

At the end of each trial, survivors were killed by decapitation and liver samples were taken. Livers were also taken from those rats that died during the experiment. Liver tissue was wet digested with nitric and sulfuric acids, and the resultant digest was analyzed for copper by atomic absorption spectrophotometry.

RESULTS

The results of those experiments in which ^{64}Cu and ascorbic acid were given to rats in various combinations of intraduodenal and intraperitoneal administration are presented in table 2. The total uptake of ^{64}Cu by several tissues (blood, heart, kidneys, and liver), and the disappearance of ^{64}Cu from the ligated duodenal segment, were used as indexes of ^{64}Cu absorption. By either criterion, ^{64}Cu absorption was depressed when ascorbic acid was put into the isolated segment along with the ^{64}Cu. When both ^{64}Cu and ascorbic acid were administered intraperitoneally, ascorbic acid did not depress uptake of ^{64}Cu by the sampled tissues. When the ^{64}Cu was given intraduodenally and ascorbic acid was given intraperitoneally, values were obtained that did not differ significantly from either those of the controls or those of the rats that received both materials intraduodenally. Thus, ascorbic acid significantly depressed ^{64}Cu absorption only when it was administered intraduodenally, along with the ^{64}Cu.

TABLE 2

Effect of ascorbic acid on the absorption of ^{64}Cu from ligated duodenal segments

Method of administration [1]		^{64}Cu uptake by sampled tissues [2,3,4]	Disappearance of ^{64}Cu from segment
^{64}Cu	Ascorbic acid		
		% of dose	*% of dose*
ID	—	17.44 [a]	46.92 [a]
ID	ID–0	11.01 [b]	26.70 [b]
ID	IP–0	15.54 [ab]	29.34 [ab]
ID	IP–2	14.48 [ab]	33.15 [ab]
IP	—	23.88 [a]	
IP	IP–0	26.88 [a]	

[1] ID = intraduodenal; IP = intraperitoneal; 0 and 2 indicate that ascorbic acid was administered simultaneously with, or 2 hours before, ^{64}Cu, respectively.
[2] Each entry is the mean of 8 observations.
[3] Sampled tissues were blood, heart, kidney, and liver.
[4] Entries in any column that are not followed by the same letter are significantly different ($P \leq 0.05$).

The effects of copper and ascorbic acid levels on weight gains, liver copper levels and mortality are indicated in table 3 which is a summary of results from four separate trials. Dietary copper level was the only treatment that had a significant effect. Liver copper level and weight gains were not affected by feeding 1% ascorbic acid, irrespective of the copper status of the rats. In each of the first three trials, mortality was higher among the rats fed the copper-deficient diet + 1% ascorbic acid than among the rats fed the copper-deficient diet. In the fourth trial, all the copper-deficient rats died regardless of whether or not they were fed ascorbic acid, so no such comparison was possible. An effect of ascorbic acid on mortality was apparent only in rats that were marginal cases insofar as survival was concerned. If the copper deficiency was mild, ascorbic acid had no effect on mortality. If the copper deficiency was too severe, as in the fourth trial, all of the copper-deficient rats died, regardless of whether ascorbic acid was fed.

The results of the experiments that measured the effect of dietary ascorbic acid on ^{64}Cu retention by copper-deficient rats from the third trial are presented in figure 1. When the ^{64}Cu was administered to the rats by stomach tube, the rats that were fed ascorbic acid retained less of the radiocopper than did those fed the basal copper-deficient diet (at all points beyond 35 hours, $P \leq 0.06$). When ^{64}Cu was administered intraperitoneally, however, there was no difference between the two

TABLE 3

Effect of dietary levels of copper and ascorbic acid on liver copper, weight gains, and mortality

Supplement	Liver copper [1]	8 week weight gain [2]	Survivors [3]
	ppm	*g*	*%*
None	2.29 [4]	233	61
Ascorbic acid, 1%	2.64	231	48
Cu, 10 ppm	15.69	277	100
Cu, 10 ppm Ascorbic acid, 1%	15.68	279	100

[1] Dry weight basis.
[2] Weight gained in the 8 weeks immediately following weaning.
[3] Percent of rats surviving the 8-week period immediately following weaning.
[4] Each entry is the mean of 36 observations.

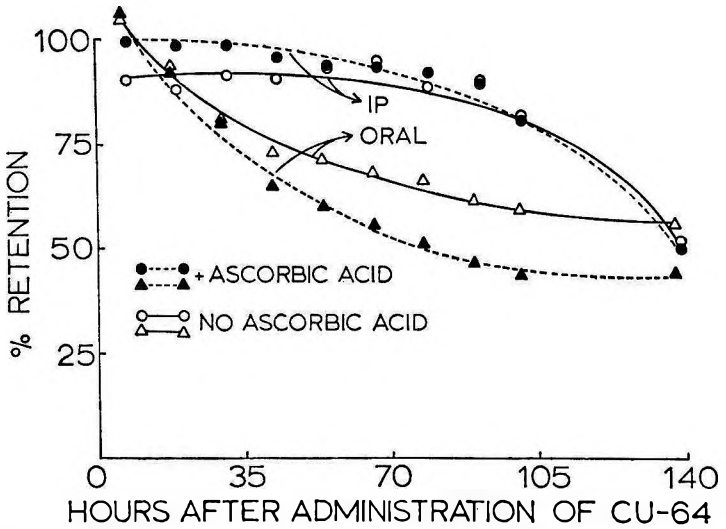

Fig. 1 Effect of dietary ascorbic acid on whole-body retention of ^{64}Cu by copper-deficient rats. (Each point is the mean of 9 observations.)

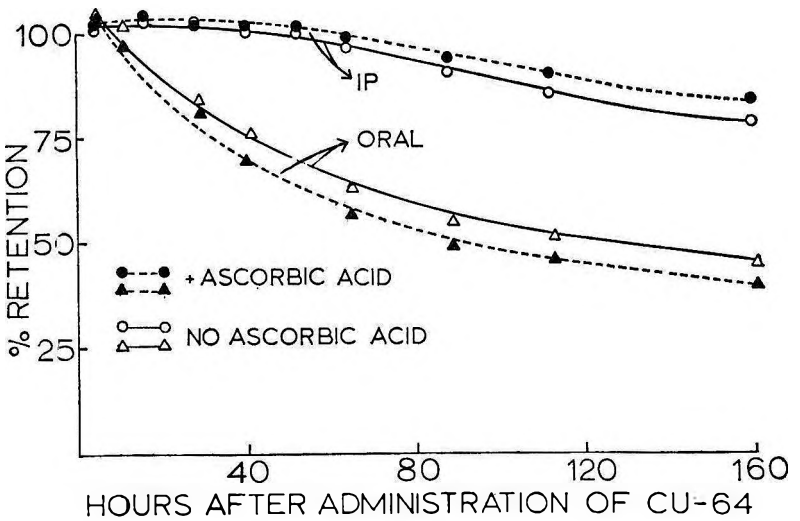

Fig. 2 Effect of dietary ascorbic acid on whole-body retention of ^{64}Cu by copper-adequate rats. (Each point is the mean of 12 observations.)

groups in their retention of the radiocopper.

The results of a similar experiment with the copper-adequate rats of trial 4 are presented in figure 2. These results are qualitatively similar to those obtained with copper-deficient rats, i.e., the rats fed ascorbic acid retained less of an oral dose of radiocopper. The differences between the rats fed ascorbic acid and the controls were not statistically significant; however, the average retention for the rats fed ascorbic acid was below that of the controls throughout the experiment. Again, when the ^{64}Cu was administered intraperitoneally, retention was as high in the rats fed ascorbic acid as in the controls.

DISCUSSION

Relatively small quantities of ascorbic acid accentuate copper deficiency in

chicks. Hill and Starcher (15) were able to depress weight gains, aortic elastin, and survival of chicks by feeding chicks ascorbic acid at the rate of 0.1% in a copper-deficient diet. Carlton and Henderson (16) observed reduced growth, lowered hemoglobin and hematocrit levels, and increased mortality when ascorbic acid was added to a semipurified diet at rates of 1 to 5 g/kg of feed. In contrast, the rats in the experiments reported here were not significantly affected by additions of 1% ascorbic acid to a copper-deficient, purified diet containing less than 1 ppm of copper. Weight gains and liver copper levels were not depressed by the added ascorbic acid, and mortality appeared to be increased only in marginal cases, i.e., it seems that for those rats that were borderline cases insofar as survival was concerned, the ascorbic acid did increase mortality. These results are consistent with those of Morris et al.,[3] and would indicate that rats are less susceptible to high levels of ascorbic acid than either chicks (15, 16) or rabbits (17).

In the experiments using ligated segments of intestine, ascorbic acid depressed the absorption of ^{64}Cu when both the acid and the radiocopper were given intraduodenally. If both were administered intraperitoneally, the ascorbic acid did not affect the uptake of ^{64}Cu by any of the tissues that were sampled. If the ^{64}Cu was given intraduodenally and the ascorbic acid intraperitoneally, absorption was intermediate between that of the controls and that of the rats that received intraduodenal ascorbic acid. These results indicate that ascorbic acid can depress the intestinal absorption of copper if the two materials are administered together. This is consistent with the report by Hunt and Carlton (17) that the administration of ascorbic acid by intramuscular injection does not increase the severity of copper deficiency in chicks.

The results with ligated segments and those of Hunt and Carlton (17) are also consistent with the data from the studies in which whole-body retention of radiocopper was measured. In these experiments, ascorbic acid apparently depressed the retention of an oral dose of ^{64}Cu, but did not depress retention of an intraperitoneal dose. If copper excretion were increased by ascorbic acid, retention of both oral and intraperitoneal doses of ^{64}Cu should be depressed when ascorbic acid is fed. However, if absorption rather than excretion is altered, the retention of the intraperitoneally administered ^{64}Cu should not be affected. This appeared to be the case in these studies. The retention experiments indicated that dietary ascorbic acid had little or no effect on excretion of ^{64}Cu and both types of studies, ligated segment and retention, demonstrated that ascorbic acid can depress the intestinal absorption of copper.

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Cellular Recovery in Rat Tissues after a Brief Period of Neonatal Malnutrition [1]

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ABSTRACT The purpose of these studies was to determine whether the known retardation in the rate of cell division in the organs of malnourished neonatal rats was reversible if adequate feeding was reinstituted during the period of active cell division. Animals undernourished for the first 9 days of life by being nursed in groups of 18 subsequently were nursed in groups of 3. The reduction in the number of cells within the organs of these animals at 9 days disappears by weaning. These data demonstrate that the cellular changes produced by neonatal undernutrition can be reversed if adequate feeding is initiated early enough.

During the neonatal period, the rate of cell division in the organs of the rat can be influenced by the state of nutrition. Decreased caloric intake curtails cell division, and the various organs contain fewer cells at weaning (1). In contrast, increased caloric intake accelerates cell division and the organs contain more cells at weaning (2). These experiments were undertaken to determine whether by increasing caloric intake to a maximum after a period of caloric restriction, the rate of cell division can be accelerated sufficiently to restore the number of cells to normal.

MATERIALS AND METHODS

Rats of the Sprague-Dawley strain were raised in litters of 18 from birth through 9 days. On day 10 all the pups were transferred to foster mothers and subsequently nursed in groups of 3. Control animals were nursed in groups of 10 for the entire period. Experimental and control animals were killed at day 9 and at weaning. Organs were immediately removed and weighed. Deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein were determined by methods previously described (3, 4).

RESULTS

1. Nine days (table 1). At 9 days, weight, protein, RNA, and DNA content of all organs are reduced. The lowered DNA content indicates that fewer cells are present; however, the "average" protein content of individual cells (protein/DNA) remains unchanged. The RNA/DNA ratio is not affected in heart, kidney, thymus, or brain, and actually increases in lung, liver, and spleen. Although brain cell number is reduced to 89% of normal, most of the reduction occurs within the cerebellum. Cerebellar DNA is reduced by 0.101 mg (72% of normal), whereas the reduction in DNA content in the rest of the brain is only 0.029 mg (97% of normal).

2. Weaning (table 2). At weaning, weight, protein, RNA, and DNA content have reached or exceed normal values in all organs except thymus. In lung, heart, liver, and kidney there is actually a significant increase in the number of cells when compared to animals nursed in litters of 10. Cell size (weight/DNA or protein/DNA) is also normal in all organs at weaning, as is the RNA/DNA ratio. The only exception to this recovery is thymus, which does not completely make up the initial cellular deficit.

If cerebellum is again examined separately, we see that the initial cellular deficit is almost completely reversed by the time of weaning (92% of normal).

DISCUSSION

Cell division in the organs of the rat ceases prior to the termination of growth.

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TABLE 1

Weight, protein, RNA and DNA content of various organs at day 9

Organ	Weight	RNA	DNA	Protein
Whole animal				
Control	19.2 ± 0.75 [1]			
Exp.	10.0 ± 1.7			
Heart				
Control	0.1113 ± 0.024	0.51 ± 0.16	0.38 ± 0.15	17.1 ± 5.2
Exp.	0.0500 ± 0.019	0.32 ± 0.13	0.22 ± 0.12	7.8 ± 2.6
Lungs				
Control	0.4940 ± 0.3	2.94 ± 0.55	4.2 ± 0.7	85.0 ± 27.8
Exp.	0.2129 ± 0.05	2.0 ± 0.75	1.5 ± 0.15	32.0 ± 3.95
Liver				
Control	0.5190 ± 0.03	8.4 ± 1.8	4.0 ± 0.1	249.1 ± 98.9
Exp.	0.3114 ± 0.07	3.5 ± 1.2	1.1 ± 0.2	72.0 ± 11.6
Kidney				
Control	0.2452 ± 0.02	1.6 ± 0.17	1.7 ± 0.05	35.1 ± 14.6
Exp.	0.1433 ± 0.02	1.03 ± 0.27	1.1 ± 0.02	21.0 ± 1.2
Spleen				
Control	0.0956 ± 0.0003	1.2 ± 0.24	1.6 ± 0.25	30.0 ± 1.6
Exp.	0.0259 ± 0.005	0.34 ± 0.09	0.26 ± 0.07	7.2 ± 2.1
Thymus				
Control	0.0603 ± 0.006	1.0 ± 0.8	0.99 ± 0.17	33.0 ± 3.20
Exp.	0.0203 ± 0.005	0.27 ± 0.09	0.31 ± 0.04	6.3 ± 1.95
Brain (total)				
Control	0.746 ± 0.03	3.40 ± 0.15	1.29 ± 0.06	62.5 ± 4.1
Exp.	0.591 ± 0.05	2.72 ± 0.05	1.16 ± 0.09	54.5 ± 2.6
Cerebellum				
Control	0.0587 ± 0.008	0.368 ± 0.07	0.361 ± 0.03	10.2 ± 0.8
Exp.	0.0365 ± 0.005	0.245 ± 0.02	0.260 ± 0.03	7.5 ± 1.7

[1] Each value represents average of 5 separate animals; ± indicates SE.

Previous experiments have demonstrated that during the period of hyperplasia, the rate of cell division can be altered by the state of nutrition (1, 2). This study demonstrates that the cellular effects produced by undernutrition are reversible if adequate feeding is instituted while cell division is still occurring. Thus, the reduced number of cells present in all organs after 9 days of caloric restriction disappears by weaning if the food deprivation is stopped and the animals are immediately "overfed." The experimental weanling rat weighs as much and his organs contain the same number of cells as weanlings from control groups.

Even if organs from experimental animals at weaning are compared with controls raised in groups of 3 from birth (2), considerable "catch up" has occurred. In animals raised in groups of 3, the total DNA content in brain at weaning was 2.54 mg (2), in animals raised in groups of 10 it was 2.45 mg, and in the rehabilitated animals in this study it was 2.41 mg. The 11% reduction in DNA after 9 days of caloric restriction, therefore, is less than 5% at weaning, even when compared to animals nursed in groups of 3 (2).

A previous study showed that refeeding ad libitum beginning after weaning would not restore the cellular deficit produced by neonatal undernutrition (1). It would appear, therefore, that the preweaning period in the rat is a critical period not only from the standpoint of the cellular effects produced by undernutrition but also from the standpoint of the possibility for those effects to be reversed.

The reason for the increase in the RNA/DNA ratio seen in certain organs after 9 days of food deprivation is not clear. This increase, however, has been

TABLE 2

Weight, protein, RNA and DNA content of various organs at weaning

Organ	Weight	RNA	DNA	Protein
Whole animal				
Control	46.9 ± 3.1 [1]			
Exp.	54.8 ± 2.9			
Heart				
Control	0.2915 ± 0.04	1.44 ± 0.7	0.992 ± 0.15	47.8 ± 5.8
Exp.	0.2770 ± 0.03	1.906 ± 0.2	1.2 ± 0.15	45.5 ± 0.4
Lungs				
Control	0.4258 ± 0.04	3.88 ± 0.7	6.28 ± 0.95	144.6 ± 49.9
Exp.	0.5187 ± 0.04	4.7 ± 0.8	8.5 ± 1.3	165.0 ± 16.5
Liver				
Control	2.0815 ± 0.2	24.0 ± 2.0	9.8 ± 3.15	511.0 ± 114.5
Exp.	2.167 ± 0.18	26.0 ± 1.4	10.0 ± 0.55	530.6 ± 20.5
Kidney				
Control	0.6208 ± 0.04	2.75 ± 1.5	2.25 ± 0.5	135.0 ± 38.3
Exp.	0.6227 ± 0.03	2.25 ± 0.65	3.45 ± 0.2	175.3 ± 12.0
Spleen				
Control	0.1814 ± 0.02	2.4 ± 0.05	3.0 ± 0.3	42.5 ± 8.35
Exp.	0.1941 ± 0.05	2.73 ± 0.15	3.3 ± 0.35	48.5 ± 8.7
Thymus				
Control	0.2495 ± 0.03	2.55 ± 0.25	7.3 ± 1.5	72.6 ± 27.5
Exp.	0.2360 ± 0.004	2.3 ± 0.1	6.4 ± 0.9	45.8 ± 8.9
Brain (total)				
Control	1.38 ± 0.04	4.95 ± 0.31	2.45 ± 0.13	266.0 ± 13.6
Exp.	1.33 ± 0.03	4.49 ± 0.14	2.41 ± 0.08	228.0 ± 40.7
Cerebellum				
Control	0.188 ± 0.03	0.736 ± 0.09	1.27 ± 0.1	27 ± 2.1
Exp.	0.184 ± 0.005	0.681 ± 0.03	1.18 ± 0.04	26 ± 2.6

[1] Each value represents average of 5 separate animals; ± indicates SE.

seen under certain other circumstances where the tissue has been exposed to various stimuli. It has been described in heart tissue during experimental cardiac hypertrophy (5), in human placenta during "placental insufficiency" (6), and in other situations (7, 8). This effect of caloric restriction seems to be an early one since it is not present in restricted animals at weaning (1).

The response in brain during these experiments may be particularly significant because 9 days of food deprivation beginning at birth has resulted not only in a reduced number of cells but also in abnormalities in lipid metabolism (9) as well as functional impairment.[2]

Animals so deprived exhibit a decrease in exploratory behavior when compared to normally nourished littermates.[2] Moreover, in man it would appear that food deprivation in the neonatal period may result in retarded development (10). It is not known whether at least part of this retardation can be reversed by early rapid institution of optimal feeding. Recent data would indicate that cell division in human brain ceases at about 5 months after birth (11). Thus, reversal of the cellular effects of malnutrition might be possible if optimal feeding is begun before that time.

Brain itself is composed of a number of regions, and there are data demonstrating that the rate of cell division and the time at which cell division stops vary from one region to another.[3] Moreover, malnutrition appears to affect these regions differently.[4] After 9 days of caloric restriction in the

[2] Barnes and Frankova 1968, personal communication.

[3] Fish, I., and M. Winick 1968 Normal regional growth. Presented at a meeting of the American Academy of Neurology (manuscript in preparation).

[4] Fish, I., and M. Winick 1968 Regional growth of brain during malnutrition. Society for Pediatric Research, Atlantic City, New Jersey, May 1 (abstract).

newborn rat the major effect is on cerebellum. These changes, however, can still be reversed if "optimal" feeding is initiated immediately. This study when coupled with previous studies (1) demonstrates not only that the quality and quantity of the food are important in recovery from malnutrition but that the time during which rehabilitative efforts are undertaken may be equally important. Return to optimal feeding will correct the cellular deficits imposed by malnutrition only if the refeeding is begun during the period of active cell division within the various organs.

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Effects of a Dietary Potassium Deficiency on Protein Synthesis in the Young Chick [1,2]

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ABSTRACT The influence of the dietary level of potassium on protein synthesis was investigated with young chicks. Incorporation of intraperitoneally injected L-leucine-1-^{14}C into TCA-precipitable material 4 hours postinjection was used as an index of rate of protein synthesis. The results indicated that chicks fed a semipurified diet deficient in potassium incorporated significantly less L-leucine-1-^{14}C into skeletal muscle protein than chicks receiving an adequate level of potassium. Conversely, the incorporation of L-leucine-1-^{14}C into plasma protein was significantly greater in potassium-deficient chicks than in chicks receiving adequate potassium. Radioactivity in the nonprotein fraction of plasma, presumably in the form of the free amino acid, was also higher in potassium-deficient chicks as compared with control chicks. Dietary potassium was without effect on incorporation of L-leucine-1-^{14}C into liver proteins. It was demonstrated, by pair-feeding experiments, that the effects on incorporation of the labeled leucine into skeletal muscle protein were due to a potassium deficiency per se rather than to reduced feed intake.

Potassium has been shown to be required for protein synthesis in cell-free systems of mammalian liver (1) and *Escherichia coli* (2,3). In both cases, ammonium ions were as effective or more effective than potassium ions. However, with intact *E. coli* cells, ammonium ions were ineffective in stimulating protein synthesis, and the intracellular ammonium ion concentration was found to be much lower than that needed for stimulation of protein synthesis in a cell-free system (2,3). In either cell-free or intact cell systems, sodium and lithium ions have not enhanced protein synthesis and, in fact, have been shown to be inhibitory (2). Thus, it has been concluded that potassium is the monovalent cation which regulates protein synthesis in the cell, and that relatively small decreases in intracellular potassium result in a reduction in protein synthesis (2,3). It has been proposed that the primary mechanism by which potassium influences protein synthesis does not involve RNA synthesis or the formation of aminoacyl-s-RNA, but rather is required for the formation of a complex of aminoacyl-s-RNA, ribosome(s) and messenger RNA (3,4).

The possibility that potassium may also affect amino acid transport has recently been investigated (5). However, it was found that increasing the extracellular potassium ion concentration decreased amino acid influx and increased amino acid efflux, whereas increasing the sodium ion concentration increased influx and decreased efflux. Therefore, it was concluded that extracellular concentrations of both sodium and potassium influence amino acid transport, but that it is the high extracellular concentration of sodium which is conducive to amino acid transport.

Further evidence for a role of potassium in protein synthesis has been afforded by the observations on the high requirements for potassium for protein repletion in protein-depleted rats (6,7), and the increased requirement for potassium with high protein diets (8).

The present investigation was conducted to determine the effect of a potassium deficiency on protein synthesis in vivo and to study possible tissue differences in response to the deficiency.

EXPERIMENTAL

White Mountain cockerels were maintained in electrically heated battery brood-

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[1] Journal Paper no. 3256, Purdue Agricultural Experiment Station, Lafayette, Indiana. This investigation was supported in part by Public Health Service Research Grant no. AM-04740 from the National Institute of Arthritis and Metabolic Diseases and by a NDEA Title IV fellowship to the senior author.

[2] Presented in part at the annual meeting of the American Institute of Nutrition, Atlantic City, New Jersey, 1966. Federation Proc., 25: 610 (abstract).

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ers in a temperature-controlled laboratory. Differing levels of dietary potassium were obtained by adding potassium carbonate at the expense of glucose monohydrate to the basal diet (table 1). Feed was offered ad libitum in all experiments except where pair-fed controls were used. In these instances, the pair-fed chicks received an amount of feed equal to that consumed on the preceding day by birds fed the potassium-deficient diet. Deionized water was supplied ad libitum throughout the experimental periods.

Chicks used for L-leucine-1-^{14}C incorporation studies were either 7 or 17 days of age. The 7-day-old chicks had received the experimental rations from 1 day of age, whereas the 17-day-old chicks were raised to 10 days of age with a ration adequate in potassium (2800 ppm) and then fed the different experimental diets for 7 days.

Birds that had received diets differing in potassium content were weighed and injected intraperitoneally with a solution of L-leucine-1-^{14}C (23.3 mCi/mmole) [4] in isotonic saline. The amount of isotope used in a given experiment was constant but this amount varied from 2 to 6 μCi among experiments depending on the age of the birds. All isotope injections occurred within a 2-hour period in the morning in all experiments. After 4 hours, blood for hematocrit determinations was drawn from the wing vein, a blood sample obtained by heart puncture using a heparinized needle and syringe and the birds were then killed by severing the neck vertebra. Tissue samples were removed and frozen immediately by placing them in glass vials on dry ice.

TABLE 1

Basal diet composition

	%
Isolated soybean protein [1]	30.00
Glucose monohydrate	54.22
Soybean oil	5.00
Cellulose	3.00
DL-Methionine	0.50
Glycine	0.30
Butylated hydroxytoluene (25%)	0.05
$CaCO_3$	1.70
$CaH_4(PO_4)_2 \cdot H_2O$	3.26
NaCl	0.50
Vitamin mix [2]	1.20
Trace mineral mix [3]	0.27

[1] Assay Protein C-1, Skidmore Enterprises, Cincinnati.

[2] The vitamin premix provided the following units of vitamins/kg of diet: vitamin A palmitate, 25,000 USP units; vitamin D_3, 1200 ICU; vitamin E (*d*-alpha tocopheryl acetate), 17.6 IU; and (in milligrams) riboflavin, 9; thiamine·HCl, 6; Ca D-pantothenate, 20; niacin, 50; pyridoxine·HCl, 8; folic acid, 2; biotin, 0.3; menadione sodium bisulfite, 2; inositol, 1000; choline chloride (70%), 2000; and vitamin B_{12}, 20 μg.

[3] The trace mineral premix provided the following reagent minerals in mg/kg of diet: $FeSO_4 \cdot 7H_2O$, 400; $ZnCO_3$, 160; $CuSO_4 \cdot 5H_2O$, 20; $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 4H_2O$, 1600; NaI, 2.2; $MnSO_4 \cdot H_2O$, 500: $CoCl_2 \cdot 6H_2O$, 2.5; and $Na_2MoO_4 \cdot 2H_2C$, 1.0.

Approximately 400 mg of tissue were weighed and placed in 50-ml stainless steel centrifuge tubes. Five milliliters of distilled water were added to each tube and the contents homogenized in a tissue homogenizer [5] for 1 minute. The samples were then washed quantitatively into 50-ml glass centrifuge tubes and an equal volume of 20% trichloroacetic acid (TCA) was added. After centrifugation at 1400 × *g* for 15 minutes, the supernatants were decanted and the protein precipitates washed twice with 10 ml of 5% TCA. The supernatant and washings were pooled in 2 studies and an aliquot was counted for ^{14}C activity. The protein precipitates were digested by adding 3 ml of a 3:1 mixture of hydroxide of Hyamine (1 M in methanol) [6] and 30% potassium hydroxide to each tube. The samples were allowed to stand for 4 hours, stirred on a test tube shaker, and heated in a water bath for 45 minutes at 60°. The samples were then cooled and 5 drops of 30% hydrogen peroxide added, dropwise, as a decolorizing agent. Samples were then reheated for 30 minutes, cooled and 1 ml of glacial acetic acid added to each. The contents of the centrifuge tubes were then washed into counting vials with three 5-ml washings of scintillation fluid.[7]

Blood samples were centrifuged at 4000 × *g* for 10 minutes and 1-ml samples of plasma analyzed for ^{14}C activity. The proteins in the plasma samples were precipitated with 1 ml of 20% TCA and washed twice with 2 ml of 5% TCA as discussed previously for tissue protein. The supernatants collected after each centrifugation were pooled in counting vials and 15 ml of

[4] New England Nuclear Corporation, Boston.

[5] Servall Omni-mixer, Ivan Sorvall, Inc., Norwalk, Connecticut.

[6] Packard Instrument Company, Inc., Donners Grove, Illinois.

[7] XDC scintillation solution: p-xylene, 1 part; *p*-dioxane, 3 parts; 2-ethoxyethanol, 3 parts; naphthalene, 8.0%; 2,5-diphenyloxazole, 1.0%; 1,4 *bis*-2-(5-phenyloxazolyl)-benzene, 0.05%.

scintillation solution [8] added. The protein precipitate was digested with 2 ml of hydroxide of Hyamine:potassium hydroxide solution and prepared as previously described.

The ^{14}C activity of all samples was measured in a liquid scintillation counter.[9] Quenching in the digested protein samples was corrected by adding an internal standard to each counting vial and recounting the sample.

All data were subjected to statistical analysis using an analysis of variance as outlined by Steel and Torrie (9). The Student Newman-Keul's test (9) was used as a mean separation procedure where appropriate.

RESULTS AND DISCUSSION

As reported elsewhere (10), growth rate was depressed severely by a potassium deficiency. In these studies, chicks fed the potassium-deficient diet for the 7-day experimental period starting at one-day old and 10 days old gained at rates of approximately 10 and 50%, respectively, of chicks fed an adequate level of potassium.

Two experiments were conducted on protein synthesis in skeletal muscle of 7-day-old birds that had been fed diets containing either 800, 1600 or 4000 ppm of potassium since one day of age. The incorporation of L-leucine-1-^{14}C into skeletal muscle protein was studied at 4 hours postinjection in these experiments since studies at hourly intervals from 3 to 6 hours postinjection indicated that the amount of activity present in the TCA precipitate was approximately the same at 3 hours compared with 6 hours, and the effects of a potassium deficiency on incorporation were similar regardless of time interval. In the first experiment, significantly more ($P < 0.01$) L-leucine-1-^{14}C was incorporated into skeletal muscle protein of chicks fed the 4000-ppm level of potassium than observed in chicks fed diets containing the lower levels of potassium (exp. 1, table 2). Statistical differences ($P > 0.05$) were not observed between chicks fed the 800- and 1600-ppm levels of potassium. In the second experiment, significantly greater ($P < 0.01$) radioactive leucine incorporation was observed with each increase in dietary potassium (exp. 2, table 2). A dietary potassium level of 1600 ppm was inadequate for maximal incorporation of ^{14}C-leucine into skeletal muscle protein of chicks in both experiments.

Three experiments were also conducted on the incorporation of ^{14}C-leucine into skeletal muscle of 17-day-old chicks that had received the experimental diets for 7 days. In experiments 3 and 4 (table 2), significantly less ($P < 0.01$) ^{14}C activity was found in the protein of skeletal muscle from chicks fed potassium-deficient diets (450 or 800 ppm) as compared with chicks fed diets adequate in potassium (2800 or 4000 ppm). No significant differences ($P > 0.05$) were observed in the ^{14}C activity of skeletal muscle protein from

[8] See footnote 7.
[9] Model 314EX (Tri-Carb) Packard Instrument Company, Inc., Donners Grove, Illinois.

TABLE 2

Influence of dietary potassium on ^{14}C radioactivity in skeletal muscle [1]

Exp. no.	7-day-old chicks			17-day-old chicks							
	Protein fraction [2]			Protein fraction [2]					Supernatant fraction [3]		
	Dietary potassium, ppm										
	800	1600	4000	450	800	2800	4000	4000 [4]	450	4000	4000 [4]
1 (8)	720 [a]	989 [a]	1926 [b]								
2 (8)	667 [a]	1013 [b]	1669 [c]								
3 (12)				—	4457 [a]	7113 [b]	7151 [b]	—			
4 (15)				1546 [a]	—	—	2569 [b]	—	680 [a]	485 [b]	—
5 (10)				954 [a]	—	—	1981 [b]	2142 [b]	425 [a]	380 [b]	397 [b]

[1] Mean radioactivity values for the number of chicks indicated in parentheses for each treatment. Means in a horizontal row within each fraction with different superscripts are significantly different ($P < 0.01$).
[2] Radioactivity expressed as cpm/100 mg fresh muscle × chick weight (g)/100.
[3] Radioactivity expressed as cpm in the TCA supernatant from 100 mg fresh muscle × chick weight (g)/100.
[4] Pair-fed an amount of diet equal to that consumed by chicks fed the deficient diet (450 ppm) on the preceding day.

birds fed the 2800 and 4000 ppm levels of potassium in experiment 3.

A fifth experiment was conducted to determine whether the decreased ^{14}C-leucine incorporation resulted from a general reduction in feed consumption rather than from a specific effect due to a potassium deficiency. One group of chicks was fed the same amount of a diet containing 4000 ppm of potassium as was consumed by the deficient chicks the previous day. Chicks fed the 4000-ppm diet either ad libitum or pair-fed incorporated significantly more ($P < 0.01$) ^{14}C-leucine than the potassium-deficient chicks (exp. 5, table 2). No significant differences ($P > 0.05$) were observed between chicks fed the potassium-adequate diet ad libitum or pair-fed with the deficient diet. These observations indicated that the reduced ^{14}C-leucine incorporation resulted from a potassium deficiency rather than from a general reduction in food consumption.

The radioactivity present in the supernatant from the TCA precipitation of skeletal muscle protein was also determined in the latter 2 experiments. Significantly more ($P < 0.05$) radioactivity was present in the supernatant from potassium-deficient chicks than from chicks fed an adequate level of potassium. The increased radioactivity in the supernatant from potassium-deficient chicks probably was a reflection of the decreased incorporation of ^{14}C-leucine into skeletal muscle protein.

In contrast with the decreased ^{14}C-leucine incorporation into skeletal muscle protein, studies on the incorporation of L-leucine-1-^{14}C into plasma proteins of 17-day-old chicks showed increased amounts of radioactivity present in the TCA precipitate from the potassium-deficient chicks as compared with chicks fed adequate levels of potassium (table 3). Plasma values for experiments 3 and 4 represent blood samples taken from the same birds used in the studies on ^{14}C incorporation into skeletal muscle protein. The increased ^{14}C-leucine incorporation into the plasma proteins of potassium-deficient chicks as compared with chicks fed adequate potassium was statistically significant ($P < 0.01$) in all experiments.

Studies conducted on the supernatant fraction of plasma showed increased radioactivity present in the supernatant from deficient chicks as compared with chicks fed the 4000-ppm level of potassium. Similar results were observed in the supernatant from skeletal muscle protein precipitation.

Two experiments each with 7- and 17-day-old chicks were conducted to study the influence of dietary potassium on L-leucine-1-^{14}C incorporation into liver proteins. Chicks were fed diets containing either 800, 1600 or 4000 ppm of potassium. Significantly less ($P < 0.05$) ^{14}C-leucine was incorporated into liver protein of 7-day-old chicks fed each decreasing level of potassium in one study (exp. 6, table 4), whereas no statistically significant differences ($P > 0.05$) were observed in the other experiment (exp. 7), although the same trend was apparent. No significant differences ($P > 0.05$) in ^{14}C-leucine incorporation into liver protein of 17-day-old chicks were observed. Birds fed the deficient levels

TABLE 3

Influence of dietary potassium on ^{14}C radioactivity in plasma of 17-day-old chicks [1]

Exp. no.	Protein fraction [2]					Supernatant fraction [3]		
	Dietary potassium, ppm							
	450	550	800	2800	4000	450	550	4000
3 (12)	—	—	23001 [a]	12564 [b]	11530 [b]	—	—	—
4 (15)	38972 [a]	—	—	—	27484 [b]	5478 [a]	—	4540 [b]
10 (14)	41251 [a]	—	—	—	31524 [b]	5670 [a]	—	4670 [b]
11 (10)	—	101477 [a]	—	—	81385 [b]	—	2363 [a]	1698 [b]

[1] Mean radioactivity values for the number of chicks indicated in parentheses for each treatment. Means in a horizontal row within each fraction with different superscripts are significantly different ($P < 0.01$ in all cases except $P < 0.05$ for experiment 4 supernatant values).

[2] Radioactivity expressed as cpm in plasma protein from 1 ml whole blood corrected for hematocrit × chick weight (g)/100.

[3] Radioactivity expressed as cpm in TCA supernatant from plasma protein in 1 ml whole blood corrected for hematocrit × chick weight (g)/100.

TABLE 4

Influence of dietary potassium on ^{14}C radioactivity in liver protein [1]

Exp. no.	7-day-old chicks			17-day-old chicks		
	Dietary potassium, ppm					
	800	1600	4000	800	1600	4000
6 (8)	1632 [a]	2025 [b]	2479 [c]	—	—	—
7 (8)	2059 [a]	2184 [a]	2346 [a]	—	—	—
8 (7)	—	—	—	15591 [a]	13347 [a]	11945 [a]
9 (7)	—	—	—	15588 [a]	15833 [a]	13739 [a]

[1] Radioactivity values are expressed as cpm/100 mg fresh liver × chick wt (g)/100 and represent the mean for the number of chicks shown in parentheses for each treatment. Means in a horizontal row with different superscripts are significantly different ($P < 0.05$).

of potassium appeared to have slightly higher amounts of radioactivity in the liver protein than birds fed the diet containing 4000 ppm potassium. Previous studies by Rinehart et al. (10) have shown that the day-old chick becomes more severely depleted of potassium after being fed the deficient diet for 7 days than the 10-day-old chick. These same studies also indicate that the liver resists depletion of its potassium content to a much greater extent than muscle or plasma. The affinity of the liver for potassium as compared with muscle and plasma may account for the less pronounced effects observed on ^{14}C-leucine incorporation in this tissue. Also, the differences in potassium depletion of the two age groups of chicks may explain the disparity in the radioactivity values observed in the liver of these 2 groups of chicks.

The reasons for the opposing effects of a potassium deficiency on ^{14}C-leucine incorporation into skeletal muscle and plasma proteins as compared with that observed in chicks fed adequate levels of potassium are not immediately obvious. It has been shown (10) that plasma potassium decreases rapidly in a potassium deficiency, followed by a reduction in the potassium concentration in skeletal muscle, whereas liver is quite resistant to potassium depletion. Skeletal muscle protein would comprise the major site of amino acid utilization by virtue of the large amount of skeletal muscle in the chick's body. A small decrease in ^{14}C-leucine incorporation into skeletal muscle protein could result in a marked increase in the specific activity of the labeled amino acid in the plasma and an increased incorporation of radioactivity into plasma proteins. It has been shown experimentally that potassium-deficient and control chicks exhibited specific activities of 233 and 119 cpm/μg leucine, respectively. Although plasma potassium decreases in the deficient chick, synthesis of plasma proteins in the liver may occur at a near-normal rate since liver potassium resists depletion. As a result of the higher specific activity in the deficient birds due to reduced synthesis of muscle protein, increased amounts of radioactivity would be observed in plasma proteins without an increase in protein synthesis. However, previous studies by Rinehart et al. (10) have shown that total plasma proteins are also significantly increased in potassium-deficient chicks. The apparent increase in incorporation of L-leucine-1-^{14}C into plasma protein may, therefore, be the result of both an increase in plasma protein synthesis and an increase in specific activity of the labeled amino acid.

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Dietary Carbohydrate and Serum Cholesterol in Rats [1]

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ABSTRACT The influence of various dietary carbohydrates on serum cholesterol in rats was studied with hypercholesterolemic diets containing a variety of carbohydrates. Experiments with sucrose, pregelatinized potato starch, glucose and fructose confirmed that animals fed sucrose had higher serum cholesterol levels than those fed starch and that animals fed glucose did not vary significantly from those fed starch. In experiments with carbohydrates of various chain lengths including 3 pregelatinized starches, 3 disaccharides and 2 monosaccharides, the shorter the chain length the higher the serum cholesterol level. Monosaccharides are an exception. The monosaccharide exception may be glucose alone, with only carbohydrates containing other than glucose units being hypercholesterolemic. With several different mixtures of starch and sucrose as the dietary carbohydrate, regression analysis of serum cholesterol showed a linear relation of significant slope. The larger the proportion of dietary sucrose, the higher the serum cholesterol.

Dietary carbohydrate reportedly has a role in regulating serum cholesterol. Portman et al. (1), studying rats fed diets containing cholesterol and cholic acid, observed that animals with sucrose in their diets had higher cholesterol levels than animals fed starch. When they compared sucrose, glucose and fructose, the hypercholesterolemic effects were equal, although glucose caused somewhat lower cholesterol levels than the other sugars. Guggenheim and co-workers (2), with more moderate cholesterol-cholic acid-containing diets, reported no differences between the serum cholesterol levels of animals fed cornstarch or sucrose, but animals fed glucose had lower levels. Lower cholesterol levels also were observed in conventionally reared chickens (3–5), and rabbits (4), eating diets containing glucose as compared with those eating diets containing sucrose. Nath et al. (6) observed that substituting wheat flour or ground wheat for sucrose in a high carbohydrate diet caused a reduction in serum cholesterol, dextrin caused no significant effect and lactose caused an increase. Wells and Anderson (7) observed that feeding lactose as compared with sucrose resulted in higher serum cholesterol concentrations in rabbits. In man the effects of dietary carbohydrates on serum cholesterol have been reviewed recently (8–10). The replacement of dietary sucrose by starch usually caused a decrease in serum cholesterol.

Our experiments were undertaken to study the effects of simple sugars as compared with starches on serum cholesterol in experimental hypercholesterolemic diets relatively high in carbohydrate. The proportion of carbohydrate, fat and protein contained in the experimental diets was based on the amounts of these nutrients available per capita per day in the United States (11).

EXPERIMENTAL

The experimental diets consisted of the following: (in percent) casein,[3] 16; hydrogenated vegetable oil,[4] 24; carbohydrate, 54.25; U.S.P. salt mix XIV, 4; adequate vitamins, listed below; cholesterol, 1; and cholic acid, 0.5. In experiment 1, cholesterol and cholic acid were omitted in 4 diets as noted in the text; otherwise the diets were identical in all respects except for the type of carbohydrate. The carbohydrates used were obtained from various commercial sources.

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[3] A.N.R.C. Casein, Sheffield Chemical Company, Norwich, New York.

[4] Primex B and C, Procter and Gamble Company, Cincinnati.

The vitamin mixture consisted of the following (in milligrams per 100 grams of diet): *p*-aminobenzoic acid, 30; biotin, 0.03; D-calcium pantothenate, 5.0; folic acid, 0.09; *i*-inositol, 20; menadione, 0.2; nicotinic acid, 9; pyridoxine hydrochloride, 2; riboflavin, 5; thiamine hydrochloride, 1.2; vitamin A acetate (325,000 USP units per gram) with vitamin D_2 (32,500 USP units per gram), 1.23; vitamin E acetate, 10; cyanocobalamin, 0.01; choline chloride 100. The mixture was made up to 250 milligrams with corn starch.

Male albino rats [5] ranging from 210 to 275 g in initial weight were used. They were assigned at random 15 per diet from blocks of animals having similar initial body weights. They were housed in individual wire-mesh-bottom, galvanized cages in air conditioned quarters kept as close as possible to 24° and 50% relative humidity. The animals were allowed food and water ad libitum. After 28 days the animals were exsanguinated in a random order following an overnight fast of 16 to 23 hours. The killing of animals took place over a 12-day period; the average time an experimental diet was fed was 34 days.

Weight gain and food consumption were measured during the first 28 days of the experiments before killing was started. During the killing period food was allowed ad libitum but neither food consumption nor weight gain was measured after day 28.

Serum cholesterol was determined by the method of Mann (12). The data were tested for significance by means of analysis of variance. In all cases where significant results are discussed, $P \leq 0.05$. The standard deviations of the cholesterol values varied directly as the means, therefore for statistical analysis, the cholesterol values were transformed to logarithms. The values presented were obtained by reconverting the data to their antilogs. The asymmetric distribution of the standard errors around their means results from the reconversion.

RESULTS

Table 1 presents serum cholesterol data from animals fed sucrose, fructose, glucose, and pregelatinized potato starch (exp. 1). The results confirm that sucrose was hypercholesterolemic compared to starch. The serum cholesterol of animals fed glucose did not vary significantly from those fed starch. Fructose eating animals had cholesterol levels which were similar to those eating sucrose. Table 1 also shows that without the addition of cholesterol and cholic acid to the diet, the cholesterol levels were essentially identical.

Table 2 contains data from a larger study (exp. 2), using a wider variety of carbohydrates. Three pregelatinized starches, three commercial dextrins, three disaccharides, and two monosaccharides were used. Analysis of variance of the serum cholesterol levels followed by paired

[5] CFE Strain, Carworth Farms, New City, New York.

TABLE 1

Effect of dietary carbohydrate on serum cholesterol (exp. 1)

Carbohydrate	Serum cholesterol	
	With cholesterol and cholic acid	Without cholesterol or cholic acid
	mg/100 ml	*mg/100 ml*
Sucrose	348^{+44}_{-41} [a] [1] (14) [2]	97^{+3}_{-5} [c] (25)
Fructose	358^{+49}_{-43} [a] (15)	99^{+8}_{-5} [c] (15)
Glucose	296^{+29}_{-28} [b] (15)	93^{+7}_{-7} [c] (15)
Pregelatinized potato starch	236^{+29}_{-26} [b] (13)	91^{+4}_{-5} [c] (25)

[1] Mean and SE. Means without a common letter in their superscripts are significantly different at $P \leq 0.05$.

[2] Number of rats/group.

TABLE 2

Effect of dietary carbohydrates on serum cholesterol (exp. 2)

Carbohydrate	Serum cholesterol
	mg/100 ml
Potato starch	258^{+32}_{-29} [a 1,2]
Wheat starch	196^{+22}_{-20} [a]
Cornstarch	188^{+22}_{-20} [a]
Starch mean	211^{+15}_{-14}
Dextrin 15 [3]	235^{+37}_{-33} [a]
Dextrin 24	232^{+28}_{-24} [a]
Dextrin 42	270^{+31}_{-28} [ab]
Dextrin mean	245^{+18}_{-17}
Sucrose	303^{+37}_{-33} [b]
Lactose	387^{+43}_{-38} [b]
Maltose	283^{+32}_{-30} [ab]
Disaccharide mean	321^{+21}_{-20} [4]
Fructose	180^{+29}_{-14} [a]
Glucose	236^{+14}_{-14} [a]
Monosaccharide mean	206^{+18}_{-16}

[1] Mean and SE. Means without a common letter in their superscripts are significantly different.
[2] Fifteen rats/group.
[3] Number following dextrin is the percent reducing sugar.
[4] Disaccharide mean is significantly higher than means of the other carbohydrate classes.

comparisons of the means of each carbohydrate class showed that the mean serum cholesterol level of animals fed the disaccharide class was significantly higher than that of the other groups. When the mean of each of the carbohydrate classes is plotted graphically (fig. 1), the shorter the chain length of the dietary carbohydrate group tested in this study, the higher the cholesterol level. Animals fed monosaccharide diets are an exception. Their mean cholesterol level is similar to the mean of the starch group.

Because the animals fed fructose in the second experiment were low in serum cholesterol compared with sucrose-fed animals whereas in experiment 1 animals eating fructose and animals eating sucrose had similar levels of serum cholesterol, another experiment was carried out including fructose. In this third study (table 3) rats fed sucrose had significantly higher cholesterol values than animals eating either pregelatinized potato starch or fructose.

In experiment 4 (table 4) we studied the effects of mixtures of carbohydrates in the diet on serum cholesterol. Mixtures of pregelatinized potato starch and sucrose were used as the dietary carbohydrate in 5 diets. The data showed the usual higher serum cholesterol levels in animals fed

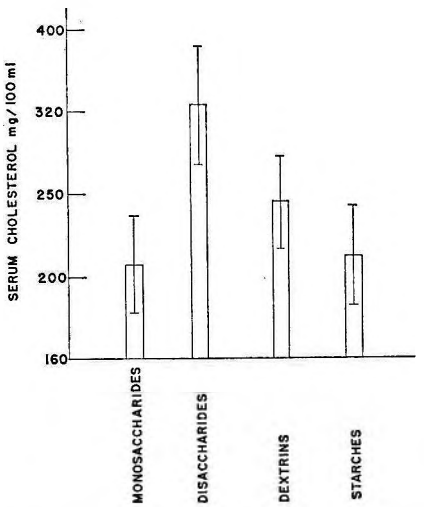

Fig. 1 Effect of the class of dietary carbohydrate on serum cholesterol. The thin vertical bars represent the SE of the mean.

TABLE 3

Effect of carbohydrates on serum cholesterol (exp. 3)

Carbohydrate	Serum cholesterol
	mg/100 ml
Potato starch	252^{+23}_{-22} [a 1,2]
Sucrose	386^{+36}_{-34} [b]
Fructose	258^{+28}_{-25} [a]

[1] Mean and SE. Means without a common letter in their superscripts are significantly different.
[2] Fifteen rats/group.

sucrose compared with those eating starch (8). Regression analysis of the data showed a linear relation of significant slope (fig. 2). There is a positive correlation between the serum cholesterol level and dietary sucrose and a negative correlation between serum cholesterol and dietary starch.

The means of the weight gain and food consumption for each group in each experiment are shown in table 5. We find when comparing animals fed pregelatinized potato starch with animals fed sucrose that although sucrose-fed animals ate slightly less and therefore had a slightly lower intake of dietary cholesterol, their serum cholesterol levels are consistently higher.

TABLE 4

Effect of dietary carbohydrate on serum cholesterol (exp. 4)

Carbohydrate [1]		Serum cholesterol
Starch	Sucrose	
%	%	*mg/100 ml*
—	100	426^{+27}_{-26} [a] [2] (13) [3]
25	75	414^{+47}_{-43} [a] (14)
50	50	333^{+37}_{-33} [b] (14)
75	25	319^{+34}_{-31} [b] (14)
100	—	313^{+26}_{-23} [b] (14)

[1] Percent of sucrose and starch contained in the carbohydrate portion of the diet.
[2] Mean and SE. Means without a common letter in their superscripts are significantly different.
[3] Number of rats/group.

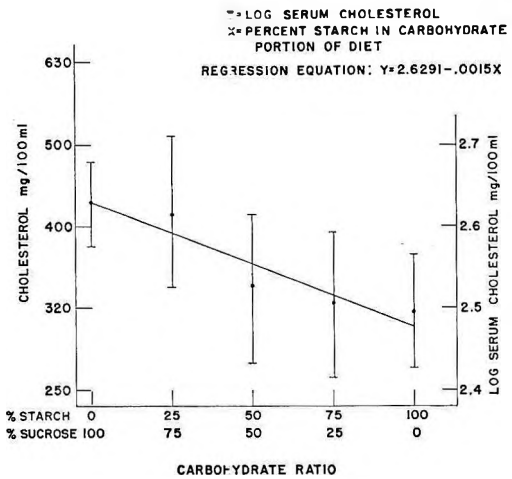

Fig. 2 Effect of several dietary combinations of sucrose and pregelatinized potato starch on serum cholesterol. The scale at the right and the regression equation are in logarithms. The thin vertical bars represent the SE of the mean.

DISCUSSION

The results of feeding sucrose, starch and dextrose agree with those reported by Portman et. al (1) using rats fed hypercholesterolemic diets, but feeding fructose caused variable results in our experiments. The cholesterol levels of animals fed fructose were similar to those fed sucrose or starch depending on the experiment. Our data also agree wth experiments using rabbits and chickens fed hypercholesterolemic diets containing dextrose, sucrose and starch (3–5). The observations of Guggenheim et al. (2) with diets containing cholesterol and cholic acid disagree in part with the results reported here. Our data consistently showed that animals eating diets containing pregelatinized starches had lower serum cholesterol levels than those eating diets containing sucrose. Their animals eating sucrose or starch had similar serum cholesterol levels, whereas only those eating dextrose had lower levels. The lowering of serum cholesterol by a wheat flour diet was credited by Nath et al. (6) to its gluten content, but our experiment showed that the lowering can take place with each of the pregelatinized starches which are nearly protein-free. Fillios et al. (13) confirmed the hypocholesterolemic effect of starch for a short period but reported the disappearance of the effect after 12 to 17 weeks. Our experiments do not contradict this since our average test period was 5 weeks. In a recent experiment, however, differences in response to dietary carbohydrate in both liver and serum cholesterol levels persisted for long periods under some experimental conditions (14).

Our results support the hypothesis that dietary carbohydrate has an influence on serum cholesterol. In the type of diet used in these experiments, disaccharides are hypercholesterolemic. Their influence as illustrated by sucrose is proportional to

TABLE 5

Weight gain and food consumption during 28-day experimental period

Exp. no.	Dietary carbohydrate	Wt gain	Food consumed
		g	*g*
1 (with cholesterol and cholic acid)	Sucrose	117	485
	Fructose	85	569
	Glucose	111	531
	Potato starch	113	571
1 (no additives)	Sucrose	121	492
	Fructose	94	591
	Glucose	155	422
	Potato starch	104	564
2	Potato starch	86	407
	Wheat starch	79	460
	Cornstarch	111	428
	Dextrin 15 [1]	80	366
	Dextrin 24	99	381
	Dextrin 42	101	447
	Maltose	99	441
	Sucrose	102	396
	Lactose	37	333
	Glucose	91	377
	Fructose	85	388
3	Sucrose	103	453
	Potato starch	122	509
	Fructose	91	523
4	100% sucrose [2]	87	545
	75% sucrose + 25% starch	97	653
	50% sucrose + 50% starch	91	610
	25% sucrose + 75% starch	82	639
	100% starch	88	706

[1] Number following dextrin is the percent reducing sugar.
[2] Percent of sucrose and starch contained in the carbohydrate portion of the diet.

their concentration in the diet, and the converse is true of gelatinized starch. Chain length may be a factor in the influence of carbohydrates. The shorter the chain length of the dietary carbohydrate the higher the serum cholesterol, but monosaccharides caused effects similar to those of starch. The monosaccharide exception to the chain length observation may be only glucose, with carbohydrates containing other than glucose units being hypercholesterolemic. The highest serum cholesterol levels in these experiments are observed in animals fed sucrose or lactose diets and in one feeding trial, with a fructose diet. If the serum cholesterol levels of animals eating diets containing fructose were consistently similar to those eating sucrose, a hypothesis that serum cholesterol is elevated by carbohydrates containing other than glucose could be supported.

Although the fructose data are inconsistent, every precaution was exercised in controlling the possibly significant variables. The fructose used was from one batch of food grade fructose. All animal groups were fed their experimental diets simultaneously. All were fed and weighed on the same days. Blood was collected from an equal number in each group on every day of killing. Cholesterol analyses also were performed on serums from an equal number of animals from each group for each set of analyses. The mean body weight of each group was nearly equal initially. The distribution of animals in the cages in the conditioned animal rooms was such that rats from each group were scattered throughout the room and in varying positions in each cage truck according to a standard pattern. When the discrepancy in serum cholesterol level of animals fed fructose was observed between

experiments 1 and 2, samples of the diets were reanalyzed to be certain that the animals in both experiments were fed fructose, and this was verified. Extreme caution on this point was exercised when the diets were prepared for the third experiment. Further experiments with fructose are indicated to investigate the inconsistency, particularly since there is some belief that fructose may cause the change in serum cholesterol and other serum lipids observed when sucrose is fed (15).

The hypercholesterolemic diet plays a role when considering the effect of dietary carbohydrate on serum cholesterol. The data indicate that without the addition of cholesterol and cholic acid to the diet the carbohydrates have similar effects on serum cholesterol. This confirms the results of Portman et al. (1) and Guggenheim et al. (2) when feeding diets without these additives.

ACKNOWLEDGMENTS

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Effects of Hormones Supplied in the Diet on Chick Growth and Bone Mineralization [1]

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ABSTRACT Studies were conducted on the effects of hormones, supplied in a purified diet moderately deficient in calcium, on growth and bone mineralization of chicks. Hydrocortisone at all levels, fed to chicks, was found in general to increase the percentage of bone mineral, calcium and phosphorus. Growth at the higher hormone levels, however, was markedly retarded. Cortisone was much less effective than hydrocortisone, and gonadal hormones in the amounts used failed to influence bone mineralization. Even when growth at the lower levels of hydrocortisone was approximately normal and bone tissue per unit body weight was unchanged, a slight increase in percentage bone mineral occurred. The percentage mineral was also increased in chicks retarded in growth by hydrocortisone in comparison with normal younger chicks of approximately the same size, but bone tissue per unit weight was decreased. The chicks receiving hydrocortisone thus had smaller but better mineralized bones. The favorable effect of hydrocortisone on the mineralization of developing bone was finally demonstrated by showing that chicks supplied the hormone had a greater percentage of bone mineral, calcium and phosphorus than chicks of the same weight and age retarded by low calorie intake. The effect of hydrocortisone on the mineralization of bone was observed only when the diet was deficient in calcium. Under these conditions a low level of the hormone appeared to compensate for the calcium deficiency.

Studies with chicks have been conducted to ascertain the effects of the dietary additions of hormones, particularly cortisone and hydrocortisone, on growth and bone mineralization. Previously Karnofsky et al. (1), Sames and Leathem (2) and Evans (3) observed that cortisone exerted an inhibitory effect on the growth of chick embryos. Experiments in vitro by Buno and Goyena (4) showed that cortisone and hydrocortisone retarded the growth of chick embryo limb buds. Montgomery (5) found that the growth of chicks, given daily subcutaneous injections of 5.0 mg of cortisone from time of hatching to 18 days of age, was markedly decreased. In contrast, Dulin (6) reported that daily subcutaneous injections of cortisone from 19 to 40 days of age, in amounts graded up to 1.0 mg, failed to retard the growth of cockerels but significantly slowed up the growth of capons. These observations have been confirmed by Chi (7). Huble (8) presented evidence that chondrogenesis in the proliferation zones of the proximal and distal ends of the leg bones of cockerels was greatly inhibited by a daily dose of 5.0 mg of cortisone acetate for 7 days, ending at 26 days of age. A dose of 1.4 mg of cortisone acetate daily for 5 days, ending at 25 days of age, however, was less growth retarding. The results of the studies presented in this report confirm and extend to some extent the findings just reviewed and provide new evidence of the effects of cortisone and hydrocortisone on growth and bone mineralization of chicks. In connection with the research on corticoidal hormones a few related experiments on gonadal hormones have also been conducted, but, since the results were not striking, they are not presented in detail in this report.

EXPERIMENTAL

The studies were conducted with Arbor Acre male chicks.[2] The chicks were placed on experiment approximately 24 hours after hatching. The duration of all experiments was 4 weeks. The hormones given the chicks were cortisone, hydrocor-

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[2] Obtained from the West Coast Division of Arbor Acre Farm, Inc., Nipomo, California.

tisone, dienestrol and testosterone. Cortisone and hydrocortisone were supplied as the acetates in amounts to provide equivalent quantities of the respective hormones. Dienestrol was furnished as the diacetate and testosterone as the propionate. The experimental diets containing the hormones were supplied ad libitum except for those in experiment 8 and tap water was given for drink. The composition of the basal diet is given in table 1. The chicks were weighed individually at the start of each experiment and weekly thereafter. A record of feed consumption was also made weekly. All treatments were duplicated, each lot containing 10 chicks. The criteria were rate of growth, weight of fat-free dry tibiae and quantity of ash, calcium and phosphorus in fat-free dry tibiae. During the growth phase of chickens the fat-free dry tibiae represent a close approximation of the total amount of calcifiable and calcified bone tissue in the tibiae. It is composed for the most part of bone matrix, consisting essentially of collagen, mucoprotein and mucopolysaccharides, and of bone mineral, made up largely of calcium phosphate salts. By subtracting the amount of bone mineral from the weight of the fat-free dry tibiae an estimation of bone matrix is obtained. Only data on final average weight, milligrams of fat-free dry tibiae (bone tissue) per gram chick weight, and percentage ash (bone mineral), calcium and phosphorus are presented in this report, since bone matrix and any other values needed in interpreting the findings obtained in the investigation can be readily derived from them. Expressing the amount of bone tissue in terms of unit body weight is done to give information on relative bone and body development.

TABLE 1

Composition of basal diet [1]

	parts
Cornstarch	55.7
Soybean protein [2]	28.0
Methionine	0.65
Glycine	0.35
Soybean oil	4.00
Soybean lecithin	1.00
Cellulose [3]	3.00
$Ca(H_2PO_4)_2 \cdot H_2O$	0.759
$CaCO_3$	1.198
Mineral mixture [4]	2.21
Vitamin mixture [5,6]	3.15
Total	100.017

[1] This diet was fed in experiments 5, 6 and 7 but is identical with the one fed in the previous experiments except that the vitamin B_{12}, choline, iron and magnesium content was increased so as to provide quantities of these nutrients in excess of the chick requirements given in National Academy of Sciences — National Research Council publ. 1345, 1966.

[2] Experiments 1–4 incl., C-1 Assay Protein, Skidmore Enterprises, Cincinnati; experiments 5–8 incl., RP-100 Assay Protein, Ralston Purina Company, St. Louis.

[3] Solka-Floc, Brown Company, Berlin, New Hampshire.

[4] When included in the diet at a level of 2.21%, the mineral mixture contributed the following mineral compounds: (g/kg) K_2HPO_4, 12.0; $MgSO_4 \cdot 7H_2O$, 6.0; NaCl, 5.0; $NaHCO_3$, 1.44; $FeSO_4 \cdot 7H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 0.308; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.25; ZnO, 0.07; $CuSO_4 \cdot 5H_2O$, 0.024; $Co(C_2H_3O_2) \cdot 4H_2O$, 0.005; KI, 0.005; NaF, 0.005; $Na_2MoO_4 \cdot 2H_2O$, 0.005; $NaSiO_3 \cdot 5H_2O$, 0.004; and $NaSeO_3 \cdot 5H_2O$, 0.00035.

[5] When included in the diet at a level of 3.15%, the vitamin mixture contributed the following vitamins: (mg/kg) thiamine·HCl, 10.0; riboflavin, 10.0; pyridoxine·HCl, 10.0; Ca pantothenate, 30.0; niacin, 100.0; folic acid, 5.0; biotin, 0.2; vitamin B_{12} (3 mg/g), 6.67; 2-methyl-1,4-naphthoquinone, 10.0; vitamin A (10,000 IU/g), 1000.0; vitamin D_3 (1500 IU/g), 1000.0; vitamin E (44 IU/g), 2000.0; and choline chloride (44%), 3410.0. The vitamins were mixed with glucose to make up the required percentage.

[6] The vitamin A and D_3 preparations were made by Hoffmann-La Roche, Nutley, N. J., the vitamin E by Distillation Products Industries, Rochester, New York and the vitamin B_{12} by Chas. Pfizer & Company, New York, New York. Vitamin A was supplied as vitamin A palmitate, vitamin D_3 as D-activated animal sterol and vitamin E as *d*-α-tocopheryl acetate.

At the end of every experiment 6 chicks (3/lot) from each treatment group were killed and the left tibia dissected. The average weight of these chicks was approximately the same as that of all chicks of the group. After removal of the muscular tissue from the tibiae, they were extracted first with alcohol for approximately 24 hours, and then with ether for 24 hours, to remove water and fat and related substances. Ashing of the fat-free dry bones was carried out in a muffle furnace at 600° for 24 hours or until no further loss of weight occurred. The calcium content of the bone mineral was determined by the atomic absorption procedure and the phosphorus content by the method of Kitson and Mellon (9).

Preceding the work with hormones, research was carried out to determine the most suitable submarginal dietary levels of calcium and phosphorus to be supplied the chicks (exp. 1). The results of this portion of the work showed that somewhat below normal growth and slightly subnormal bone mineralization under the experimental conditions were obtained when the diet contained 0.6% calcium and

0.4% phosphorus. Therefore, unless indicated otherwise, these levels of calcium and phosphorus were supplied in the subsequent work in an effort to get a more adequate conception of the effect of hormones on growth and bone mineralization.

RESULTS AND DISCUSSION

In the first experiments with hormones (exps. 2 and 3), the results showed that dienestrol at 50 to 250 mg/kg diet failed to influence growth or bone mineralization but testosterone appeared to retard growth slightly at 25 mg/kg and greatly at 250 mg. No consistent effect on bone mineralization, however, was observed. In subsequent experiments the levels of estrogen and androgen used were 50 mg/kg and 25 mg/kg, respectively.

The work with cortisone showed that 25 to 125 mg/kg diet had no detrimental effect on either growth or bone mineralization. Cortisone at 350 mg/kg, however, appeared to retard growth somewhat (8.1%), but the amount of bone tissue per unit weight and the percentage of mineral and calcium in the tibiae were increased slightly (table 2, exp. 3). When hydrocortisone was fed at 350 mg/kg diet in place of cortisone, growth was severely retarded and only 6 of the chicks survived. The percentage of mineral, calcium and phosphorus in the tibiae, however, was increased slightly, but milligrams of bone tissue per gram of final chick weight were greatly reduced. On comparing these values with those of 7-day-old chicks of only slightly greater physiological size, it appeared that hydrocortisone promoted a decrease in percentage bone matrix and an increase in percentage bone mineral. This is believed to be an indication that hydrocortisone exerts 2 apparently opposite effects; 1) the well-known deterrent effect on protein synthesis, including synthesis of bone tissue protein, and 2) an effect, either direct or indirect, favoring bone mineralization (table 2, exp. 3 vs exp. 2). All chicks fed hydrocortisone in combination with either estrogen or androgen, or both, died during the course of the experiment.

When the percentage bone matrix and percentage bone mineral in the bone tissue per gram weight of the 7-day-old normal chicks and the 28-day-old chicks retarded by hydrocortisone was calculated, the amount of bone mineral per gram weight of the latter was only 3.0% less than the former, but the amount of bone matrix per gram weight was 23.1% less. Thus, the chicks retarded in growth by hydrocortisone had much smaller bones than the 7-day-old normal chicks but the difference was due largely to lower matrix content.

When hydrocortisone levels of 20 to 100 mg/kg diet in variations of 20 mg were fed with, and without, estrogen and androgen at levels of 50 mg and 25 mg/kg diet, respectively, all quantities of hydrocortisone markedly retarded growth (table 3, exp. 4). Because of the marked effect of 20 mg of hydrocortisone per kilogram diet on growth, the analytical work on the tibiae of the chicks given the higher levels of hydrocortisone was omitted. The percentage mineral, calcium and phosphorus in the bone tissue of the tibiae was found to be somewhat greater when hydrocortisone was supplied but the bone tissue per gram of final chick weight was consider-

TABLE 2

Effect of corticoidal hormones on growth and bone development [1] *(exps. 2 and 3)*

Experiment no.		Age, days	Avg body wt	Bone tissue	Amount in bone tissue of tibiae		
					Minerals	Calcium	Phosphorus
			g	*mg/g wt*	%	%	%
2	No hormones	7	81	2.53	34.3	13.8	6.67
2	No hormones	28	425	3.51	39.0	14.0	7.70
3	No hormones	28	434	3.32	38.7	11.6	6.47
3	Cortisone [2]	28	399	3.47	39.4	12.4	5.95
3	Hydrocortisone [3]	28	69	2.12	39.7	15.4	6.83

[1] Diet contained 0.6% calcium and 0.4% phosphorus.
[2] Cortisone, 350 mg/kg diet.
[3] Hydrocortisone, 350 mg/kg diet.

TABLE 3

Effect of hydrocortisone, testosterone plus dienestrol and all hormones combined on growth and bone development [1] *(exp. 4)*

	Treatment	Avg 28-day wt	Bone tissue	Amount in bone tissue of tibiae		
				Minerals	Calcium	Phosphorus
		g	*mg/g wt*	%	%	%
1	No hormones	459	3.56	37.4	13.3	6.23
2	Hydrocortisone [2]	211	2.81	39.4	16.3	7.56
3	Gonadal hormones [3]	452	3.66	37.3	14.0	6.35
4	Hydrocortisone + gonadal hormones	210	3.02	39.6	15.3	6.95
Avg 1 and 3		455	3.61	37.4	13.7	6.29
Avg 2 and 4		211	2.91	39.5	15.8	7.26

[1] Diet contained 0.6% calcium and 0.4% phosphorus.
[2] Hydrocortisone, 20 mg/kg diet.
[3] Gonadal hormones, 25 mg testosterone/kg diet; 50 mg dienestrol/kg.

TABLE 4

Results of supplying graded amounts of hydrocortisone on growth and bone development [1,2] *(exp. 5)*

Hydrocortisone	Avg 28-day wt	Bone tissue	Amount in bone tissue of tibiae		
			Minerals	Calcium	Phosphorus
mg/kg diet	*g*	*mg/g wt*	%	%	%
0.0	451	3.69	38.5	14.0	6.80
1.25	440	3.32	39.4	14.3	7.06
2.50	446	3.60	39.7	14.4	7.12
5.00	427	3.52	40.2	14.6	7.21
10.00	336	3.37	40.9	14.8	6.92
20.00	221	2.75	41.7	15.5	7.56

[1] Diet contained 0.6% calcium and 0.4% phosphorus.
[2] Data are averages of groups with and without testosterone 25 mg/kg diet; dienestrol, 50 mg/kg.

ably reduced. The favorable effect of hydrocortisone on bone mineralization, however, is in agreement with the results of experiment 3. Growth and bone mineralization were not influenced, either favorably or unfavorably, by supplying gonadal hormones alone, or with hydrocortisone.

In further work, because of the marked growth-retarding effect of 20 mg hydrocortisone/kg of diet, levels of 1.25, 2.5, 5.0, 10.0 and 20.0 mg of the hormone per kilogram diet were fed with, and without, 50 and 25 mg of estrogen and androgen per kilogram, respectively (table 4, exp. 5). When 1.25, 2.5 and 5.0 mg hydrocortisone/kg diet were supplied, growth was almost normal but a marked reduction was obtained at 10 and 20 mg/kg. The combination of hydrocortisone with gonadal hormones may have retarded growth to a slightly further extent but this effect was not consistent.

The percentage mineral, calcium and phosphorus in the bone tissue of the tibiae was progressively increased and percentage bone matrix decreased with increased intake of hydrocortisone while milligrams of bone tissue per gram weight were unchanged except at 20 mg/kg diet, where they were markedly decreased. The increased bone mineral at 1.25, 2.5 and 5.0 mg hydrocortisone/kg diet is indicative of an effect of hydrocortisone favoring bone mineralization, since milligrams of bone tissue per gram final weight were not decreased. At these levels of hydrocortisone the increased mineralization of bones of approximately the same size, as indicated by bone tissue per gram weight, is believed to be evidence that the chicks in these groups had stronger bones. The decreased bone tissue per gram weight at 20 mg of hydrocortisone and the increased bone mineralization are in agreement with the results obtained in the previous experiment (table 3, exp. 4).

The results of experiment 5, showing that small amounts of hydrocortisone im-

proved bone mineralization, are contrary to the generally accepted hypothesis that the hormone promotes bone demineralization. Evidence in confirmation of the hypothesis has recently been reported by Eisenberg et al.(10). They found that a relatively greater than normal urinary loss of calcium occurred on direct administration of 17-hydroxycorticosteroids. In contrast, the work of Pace et al. (11), Pugh (12) and Simpson (13) points to the possibility that in prolonged muscular work and other stress conditions the increased production of 17-hydroxycorticosteroids by healthy human beings may be beneficial rather than harmful. These research workers did not study the effect of the increased 17-hydroxycorticosteroid production on bone integrity but, since this increase appears to be normal under increased muscular stress, it is possible that the difference between the effects of directly administered hormones and the increased hormone production due to prolonged physical activity is a quantitative one. Thus, the effects obtained in chicks with small quantities of hydrocortisone, and the effects obtained in human beings under stress conditions, may be similar.

Following the research with reduced amounts of hydrocortisone, graded levels of calcium were supplied with, and without hormones, in a further attempt to show that hydrocortisone exerts a favorable effect on bone mineralization during growth (table 5, exp. 6). The level of hydrocortisone provided was 5 mg/kg diet. This level of hydrocortisone did not significantly depress growth in the previous experiment. In this experiment improvements were also made in the vitamin and mineral mixtures included in the diet. The dietary changes were found to increase growth markedly, whereas contrary to expectation, the level of 5 mg hydrocortisone/kg diet greatly depressed growth. Despite this, hydrocortisone increased bone mineral at the 0.4% level of calcium to such an extent that it was equal to that obtained at 0.6% calcium without addition of the hormone. At the adequate levels of calcium, 0.8 and 1.0%, the added hydrocortisone failed to improve bone mineralization, thus indicating a calcium sparing effect at the deficient calcium levels. This is believed to be further evidence that hydrocortisone has a favorable effect on bone mineralization but only when calcium is deficient. Bone tissue per gram weight in the presence of hydrocortisone was reduced at all levels of calcium, but quantity of bone matrix was affected to a greater extent than amount of bone mineral at the two lower calcium levels. The results of this experiment, therefore, support those obtained in the previous experiment which indicated that levels of hydrocortisone that do not retard growth appear to compensate for the stress of partial calcium deficiency.

In the next experiment levels of hydrocortisone of 1.25, 2.50, 5.00 and 10.00

TABLE 5

Results of supplying graded amounts of calcium without and with hormones on growth and bone development (exp. 6)

Calcium	Avg 28-day wt	Bone tissue	Amount in bone tissue of tibiae		
			Minerals	Calcium	Phosphorus
%	g	mg/g wt	%	%	%
		No hormones			
0.4	460	3.43	31.8	10.4	5.47
0.6	559	3.64	37.1	11.8	5.44
0.8	622	4.01	40.3	13.0	6.93
1.0	608	4.18	39.3	12.2	6.64
		Hydrocortisone and hydrocortisone plus gonadal hormones [1]			
0.4	342	2.99	37.0	12.5	6.39
0.6	376	3.17	39.1	13.1	6.73
0.8	385	3.38	38.9	13.0	6.70
1.0	396	3.58	40.4	13.6	7.00

[1] Hydrocortisone, 5 mg/kg diet; gonadal hormones, 25 mg testosterone/kg; and 50 mg dienestrol/kg.

TABLE 6

Comparison of effect of retarded growth due to hydrocortisone with normal chicks of same size but younger age [1] *(exp. 7)*

Hydrocortisone	Age, days	Avg body wt	Bone tissue	Amount in bone tissue of tibiae		
				Minerals	Calcium	Phosphorus
mg/kg diet		*g*	*mg/g wt*	%	%	%
0.0	1	39	2.32	28.5	10.5	5.10
0.0	17	256	3.12	34.5	12.0	6.38
0.0	21	326	3.47	34.0	12.9	5.95
0.0	24	422	3.60	32.5	11.6	5.97
0.0	28	536	3.67	34.2	13.0	6.15
1.25	28	515	3.53	34.6	13.6	6.22
2.50	28	474	3.45	36.5	14.1	6.62
5.00	28	376	3.43	38.4	14.2	6.69
10.00	28	285	2.79	39.8	15.4	7.19

[1] Diet contained 0.6% calcium and 0.4% phosphorus.

TABLE 7

Comparison of effects on bone development of retarded growth due to hydrocortisone and comparable retarded growth due to low calorie intake [1] *(exp. 8)*

Treatment	Avg 28-day wt	Bone tissue	Amount in bone tissue of tibiae		
			Minerals	Calcium	Phosphorus
	g	*mg/g wt*	%	%	%
5 mg HC [2]/kg diet	426	3.39	38.5	14.4	6.48
LCI [3] equiv 5 mg HC	424	3.40	36.8	14.1	6.75
10 mg HC/kg diet	293	3.10	39.1	16.0	6.99
LCI equiv 10 mg HC	294	3.25	35.9	14.0	6.08

[1] Diet contained 0.6% calcium and 0.4% phosphorus.
[2] HC = Hydrocortisone.
[3] LCI = Low calorie intake.

mg/kg diet were supplied (table 6, exp. 7). The chicks in the basal group were weighed and sampled for bone development and bone mineralization twice weekly. This was made possible by increasing the number of chicks in this group at the start of the experiment. The purpose was to compare the chicks retarded in growth by hydrocortisone with chicks of the same weight but younger in age, to find out if the retarded chicks had a greater degree of bone mineralization than the normal chicks. The results showed that the chicks retarded in growth by hydrocortisone had a greater percentage of bone mineral, calcium and phosphorus in their tibiae, with the exception of the chicks given 1.25 mg of the hormone per kilogram diet, than normal chicks of approximately the same physiological size. Bone tissue per gram body weight in contrast was decreased, but was marked only at the highest hormone level. Relatively, therefore, the chicks retarded in growth with hydrocortisone had smaller bones than chicks of the same size but younger in age. The percentage bone mineral, calcium and phosphorus was also greater in the groups given hydrocortisone, with the exception of the lowest level of the hormone, than in normal chicks of the same age, but bone tissue per gram weight was considerably smaller. These results provide evidence of a favorable effect of hydrocortisone on bone mineralization when the calcium content of the diet is deficient. In work with rats, Yeager and Winters (14) observed that the percentage ash and calcium in the bones of rats, stunted by calorie, protein or lysine deficiency, was greater than in normal animals of the same weight but less than these quantities in rats of the same age.

To provide final demonstration that hydrocortisone improved mineralization in developing bone of chicks fed diets deficient in calcium, a paired-growth experiment was undertaken (table 7, exp. 8). In this experiment groups of chicks fed the basal diet were restricted in caloric intake

to such an extent as to make them grow at the same rate as groups of chicks supplied 5 and 10 mg hydrocortisone/kg diet. The results showed that at both levels of hydrocortisone, percentage of bone mineral and calcium were greater than the quantities found in the chicks fed the basal diet only. Higher percentage phosphorus, however, was found only in the pair receiving 10 mg hydrocortisone/kg diet. At 5 mg hydrocortisone/kg diet the difference in percentage of bone mineral was only 4.62% but at 10 mg hydrocortisone/kg, a more marked difference in bone ash of 8.91% was observed. Bone tissue per milligram chick weight, however, was approximately the same in each pair of chicks. The percentage of bone matrix in the tibiae was reduced, therefore, in the groups of chicks receiving hydrocortisone. This again provided evidence of the deterrent effect of hydrocortisone on synthesis of bone protein and the favorable effect on bone mineralization.

The results of the experiments presented in this report indicated that a correlation existed between percentage of bone mineral and quantity of hydrocortisone in the diet. This was clearly revealed by plotting the regression of the percentages of bone mineral on the logarithms of the quantities of hydrocortisone per kilogram diet. A linear curve was thus obtained. On calculating the correlation coefficient of the percentages of bone mineral and quantities of hydrocortisone, the correlation between these values was found to be very good ($r = 0.821$). It was also found, on applying analysis of variance to the results, that highly significant differences ($P < 0.01$) existed in the increases in percentage of bone mineral.

Similar calculations were likewise made of the effects of quantity of hydrocortisone on final average chick weight. The retarding effect of hydrocortisone on growth was highly significant ($P < 0.01$), and the inverse correlation between the final average chicks' weights and the quantities of hydrocortisone per kilogram diet was excellent ($r = 0.939$).

The increase in the percentage of bone mineral on addition of small amounts of hydrocortisone to the diet (exp. 5, table 4) was not caused by increased consumption of feed, thereby increasing calcium intake and remedying the moderate deficiency of calcium in the diet. In this experiment the feed per gain of the group supplied no hydrocortisone was 1.68 g in comparison with 1.66 g for the group that received 1.25 mg hormone/kg diet and 1.65 g for the group that received 2.50 mg hormone/kg. For the smaller quantity of hydrocortisone the increases in the amounts of bone mineral, calcium and phosphorus were, respectively, 2.34, 2.14 and 3.82% and for the higher quantity of the hormone these values were 3.12, 2.86 and 4.70%. The final average weights of the groups receiving these small amounts of hydrocortisone were almost equal to the group receiving no hormone, and bone tissue per gram final chick weight was approximately the same.

At the higher levels of hydrocortisone fed in experiment 5, however, where growth was markedly retarded, feed intake per gram gain was greatly increased. This is explained by the increased requirement of energy for maintenance relative to the growth requirement. In experiment 5, when 20 mg hydrocortisone/kg diet were supplied, the feed per gram gain was 2.52 g as compared with 1.68 g for the group receiving no hormone. At this high level of the hormone, greater increases in the amount of bone mineral, calcium and phosphorus were obtained than at the low levels. These were, respectively, 8.31, 10.71 and 9.33%. In view of the results obtained in experiment 5 at 1.25 mg and 2.5 mg hydrocortisone/kg diet it seems highly improbable that the increases in bone mineral, calcium and phosphorus were caused by increased calcium intake.

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Activity, Concentration, and Lumen-Blood Electrochemical Potential Difference of Calcium in the Intestine of the Laying Hen [1]

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ABSTRACT The driving forces for the intestinal absorption of calcium, consisting of the chemical and electrical gradients between the intestinal lumen and circulation, were evaluated in the laying hen. Those forces were related to the in vivo calcium absorption. The concentration and activity of calcium in ultrafiltrates made from the intestinal contents, were found to be dependent on dietary calcium level and source. Of the calcium sources tested, calcium sulfate supported the highest concentration and activity of calcium in the various intestinal segments. Only part of the variation in those parameters could be explained on the basis of corresponding variations in intralumenal pH. Comparison of the fraction of active calcium (activity/concentration) in the ultrafiltrates, with the theoretical activity coefficient, suggested that most of the ultrafilterable calcium in blood plasma was ionic, but that a considerable portion of the ultrafilterable calcium in the intestine was in a complexed form. The transmural electrical potential (PD) in the laying fowl was similar to that of other animals; it was lowest in the lower jejunum and highest in the colon. The electrochemical potential difference (ECPD) of calcium was calculated from the PD and the activities of blood plasma and intestinal contents. Due to a lower activity of calcium and a high PD in the lower ileum and colon, the ECPD was unfavorable for absorption of calcium in those segments as compared with the duodenum and jejunum. This coincided with an absence of any net calcium absorption in those posterior segments. The differences in the ECPD, could only partially account for the difference among the segments in the in vivo calcium absorption. The possibility of absorption of complexed calcium is discussed.

Previous studies (1, 2) have shown that the greater part of the intestinal absorption of calcium in the laying fowl occurs in the duodenum and jejunum, rather than in the ileum. At least 3 possibilities should be considered in any attempt to explain why the rate of absorption per unit length is greater in the duodenum and jejunum than in the ileum: (a) that the electrochemical potential difference of calcium is less favorable for its absorption in the ileum, (b) that the permeability of the ileal mucosa is lower than in the duodenum and jejunum and (c) that active transport of calcium is more pronounced in the upper segments, as shown in the rat, using in vitro techniques (3).

The electrochemical potential difference is a function of the mucosa-blood electrical potential difference and the activity of calcium in the intestinal lumen and blood plasma.

The transmural electrical potential difference (PD) has been studied in a number of animals (4, 5). The serosa was always positive with respect to the mucosa, hence opposing cation absorption. The PD has not been studied in the intestine of the laying hen and to our knowledge no information is available on the activity of calcium in the intestinal lumen. There are some data on the concentration of ultrafilterable calcium in the intestinal lumen of the chick (6), dog (7) and sheep (8, 9). The latter values were used to estimate the electrochemical potential difference of calcium in sheep (10), disregarding the possibility of a difference in

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activity coefficient of calcium between the intestinal contents and blood plasma.

It was, therefore, the purpose of this study to evaluate the activity of calcium in the intestinal lumen, and the transmural electrical potential difference in the laying fowl in vivo, in an effort to correlate the electrochemical potential difference of calcium with its absorption at the various levels of the intestine.

METHODS

Animals. White Leghorn laying hens, 8 to 15 months old, were used in all experiments. They were kept in individual laying cages and were maintained before the experiments with a layer diet (table 1). In trials 1 and 2, the hens were continued with the same diet. In trial 3, the respective diets (table 1) were fed 3 to 5 days before the measurements were taken.

For the experiment, the birds were killed by an overdose of sodium pentobarbital. Ligatures were placed to separate the various intestinal segments, and the intestine was immediately removed. The duodenum could be identified as the segment enclosing the pancreas, and the lower ileum, by the attached ceca. Meckel's diverticulum was taken as the point of demarcation between the jejunum and the ileum.

Preparation of ultrafiltrates. A length of dialysis tubing (Visking tubing 24/32) was washed in distilled water and the excess water was removed on filter paper. Sections of about 20 cm of this tubing were inserted into 5-ml plastic syringe barrels and inflated. The test material was introduced into the dialysis bag, and the upper portion of the bag was tied to the lip of the barrel to prevent any downward sliding of the bag. The syringe with the filled dialysis bag was placed in a 17-mm test tube, gassed with a 95% N_2 – 5% CO_2 mixture, and sealed with a double layer of Parafilm. The tube was centrifuged at 2000 rpm for 15 minutes and the resulting small quantity of ultrafiltrate was discarded. The sample was then re-gassed, sealed, centrifuged again for one hour at the same speed, and the ultrafiltrate was taken for analysis.

Ultrafiltrates were made from blood plasma and intestinal contents. The latter was directly transferred from the intestine into the dialysis bag.

Chemical procedures. Calcium was determined in the ultrafiltrates and blood plasma by a direct EDTA titration with Calcein as an indicator, under ultra violet light. Total intestinal calcium was determined by EDTA titration following oxalate precipitation as previously described (12).

For determination of phosphate, an aliquot of the ultrafiltrate was ashed in sulfuric acid and hydrogen peroxide. Phosphorus was then determined by the micromethod of Chen et al. (13).

Measurement of calcium activity. Calcium activity was measured with an Orion calcium electrode. A saturated KCl-agar bridge connected through a saturated KCl solution to a calomel electrode was used as the reference electrode. The potential difference was measured with a Philips millivoltmeter or Metrohm compensator in trial 2 and with a Keithley Electrometer

TABLE 1

Composition of experimental diets

	Trials 1 and 2	Trial 3		
		Diet 1	Diet 2	Diet 3
	% of diet	*% of diet*	*% of diet*	*% of diet*
Constant ingredients [1]	62.55	62.55	62.55	62.55
Limestone	8.00	2.11	—	—
Dicalcium phosphate [2]	1.30	1.30	4.30	1.30
Calcium sulfate [3]	—	—	—	3.46
Milo	28.15	34.04	33.15	32.69
Ca content, analyzed, %	3.56	1.31	1.30	1.33

[1] Soybean oil meal (45% protein), 20.00; yellow corn, 30.00; fish meal, 3.00; wheat bran, 5.00; soybean oil, refined, 1.00; alfalfa meal, dehydrated, 3.00; vitamin mixture, 0.25; salt and trace minerals, 0.30. For composition of vitamin and mineral mixtures, see Hurwitz and Bornstein (11).
[2] Feed grade, Chemicals and Phosphates, Haifa, Israel.
[3] $CaSO_4 \cdot 2H_2O$, precipitated; E. Merck AG, Darmstadt, Germany.

model 610B in trial 3. With either instrument, the electrode was calibrated against standard $CaCl_2$ solutions. For this standardization, the activity of the solutions was calculated using the activity coefficients given in the literature (14). In most cases, the function of the potential reading against the log of calcium activity followed Nernstian behavior. In trial 2 the results obtained with the Philips and the Metrohm instruments were identical.

The electrode was also calibrated in solutions containing sodium and potassium in concentrations similar to those determined in the test materials.

For activity measurements in plasma, blood samples were withdrawn in heparinized plastic syringes and transferred into a closed compartment containing the calcium electrode and the reference agar bridge. A stable reading was then recorded and the EMF was converted into calcium activity in millimoles/liter from the standard curve. The calcium activities were then averaged.

For measurements in intestinal ultrafiltrates, samples of about 0.2 ml were placed in a plastic dish (provided by the manufacturer), and the electrode and reference were dipped into it. Care was taken to avoid any capture of air at the tip of the electrode. The EMF readings were again converted into concentration units and averaged.

Measurement of pH. Measurements were made with a Radiometer pH meter with an expanded scale. In trial 1, the 2 electrodes — glass and calomel — were used, but in trial 3 a combined microelectrode was used. An incision was made at the left side of the abdomen of birds anesthetized with sodium pentobarbital and the electrode was inserted into the respective intestinal segment through a small puncture in its wall. The pH recorded was the average of 3 separate readings taken at different positions along the intestinal segment.

Electrical potential difference (PD) measurements. The birds were anesthetized and their abdominal cavity was exposed as above. A KCl-agar bridge was inserted into the brachial vein and secured by a ligature. A small puncture was then made in the wall of the respective intestinal segment, avoiding major blood vessels, and a second KCl-agar bridge was inserted 5 to 7 cm into the intestine, and held in place by a clamp. The intestinal segment was then returned into the abdominal cavity and the skin was clamped above the incision. The agar bridges were connected to the Philips millivoltmeter through saturated KCl solutions and matched calomel electrodes. The electrical potential was recorded for 10 minutes. The potential between 2 and 10 minutes was usually stable.

RESULTS

Trial 1. This trial had been conducted before the calcium electrode became available and was designed to study the con-

TABLE 2

The pH, electrical potential difference and ultrafilterable calcium in the intestine of the laying fowl

Intestinal segment	pH [1]	PD [2]	Ultrafilterable Ca	
		mV	*mg/100 ml*	*% of total Ca* [3]
Duodenum	6.90 ± 0.15 [4]	11.6 ± 3.0	71.0 ± 25.1	21.17 ± 13.51
Jejunum, upper	6.91 ± 0.13	9.2 ± 2.4	61.4 ± 17.1	6.00 ± 3.43
Jejunum, lower	6.95 ± 0.39	6.0 ± 1.8	72.2 ± 27.5	5.33 ± 0.23
Ileum, upper	7.80 ± 0.23	8.2 ± 1.7	31.1 ± 10.8	1.58 ± 0.52
Ileum, lower	8.01 ± 0.20	11.6 ± 4.3	24.0 ± 11.3	0.92 ± 0.10
Colon	7.40 ± 0.20	18.7 ± 4.2	44.9 ± 32.4	2.01 ± 1.09

[1] In vivo pH, measured by inserting microelectrodes in the intestinal lumen.
[2] In vivo potential measured between KCl bridges inserted in the brachial vein and the intestinal lumen, respectively. The positive sign indicates that the blood was positive with respect to the intestinal lumen.
[3] Ultrafilterable Ca as a percentage of total calcium was calculated by multiplying the concentration of ultrafilterable calcium by the total moisture content and 100, and dividing by the total calcium of the sample.
[4] Average from 5 to 7 hens ± SD.

centration of calcium in ultrafiltrates from intestinal digesta, and the fraction of the ultrafiltrable calcium in the total calcium of the intestine. This trial also included measurements of the in vivo pH and the transmural PD; its results are given in table 2.

The pH was slightly acid in the duodenum and jejunum but became considerably basic in the ileum. Although the pH decreased somewhat in the colon, it still remained slightly on the basic side.

The concentrations of ultrafilterable calcium were equally high in the 3 anterior segments of the intestine but lower at the posterior segments. The percentage of ultrafilterable calcium was calculated by multiplying the ultrafilterable calcium concentration by the total moisture and 100 and dividing by the total calcium of the gut segment. In the intestine, the non-ultrafilterable calcium appeared to be mostly in insoluble salts and the percentage of ultrafilterable calcium would, therefore, be close to the percentage of solubility. The percentage of ultrafilterable calcium was very high in the duodenum and progressively decreased with the distance from the pylorus; a small increase was noted from the lower ileum to the colon.

The PD decreased from about 12 mV in the duodenum to about 6 mV in the lower jejunum. It then progressively increased and reached about 19 mV in the colon.

Trial 2. In this trial the relationship between the calcium activity and concentration in the ultrafiltrates was studied in the blood plasma and intestinal contents. Laying hens fed the high-calcium diet (diet 1, table 1) were taken for the measurements. The results are presented in tables 3 and 4.

In general, the concentration of ultrafilterable calcium was similar to that in trial 1 except that it was higher in the upper ileum and lower in the colon. No explanation can be offered for this difference in the upper ileum. In the colon, however, a considerable quantity of urates was observed in the contents collected in trial 1. In the second trial, only the upper two-thirds of the colon was taken and no urates were observed. This suggests a leak of urine into the lower colon. Since the urine is considerably acid and contains large amounts of calcium (15), the leak accounts for the ultrafilterable calcium of the colon measuring higher in the first trial and lower in the second.

The activity of calcium (table 3) was highest in the lower jejunum and then decreased in the ileum and colon.

The fraction of active calcium (table 3) is defined as the ratio of calcium activity to ultrafilterable calcium; it was lower in all intestinal segments than the theoretical activity coefficient of 0.58 calculated on the basis of the ionic strength of the ultrafiltrates. The fraction of active calcium was highest in the duodenum and the upper jejunum. It then significantly decreased ($P < 0.05$) and reached the lowest value in the colon.

The total plasma calcium (table 4) was higher in the laying hen than that re-

TABLE 3

Partition of ultrafilterable calcium in the intestinal contents of laying hens

Intestinal segment	Ultrafilterable Ca [1]	Ca activity [2]	Active fraction [3]
	mmoles/liter	*mmoles/liter*	
Duodenum	15.9± 6.6 [4]	4.48±2.13	0.291±0.100
Jejunum, upper	12.3± 6.8	3.79±2.18	0.289±0.159
Jejunum, lower	25.6±12.4	6.69±3.47	0.252±0.055
Ileum, upper	17.6± 9.2	3.55±2.38	0.202±0.056
Ileum, lower	8.9± 3.4	1.59±0.71	0.179±0.057
Colon	8.2± 3.9	1.17±0.49	0.172±0.100

[1] Ultrafiltrates were obtained from intestinal contents by centrifugal force.
[2] Calcium activity was measured with an Orion calcium electrode.
[3] Active fraction = a_{Ca}/C_{Ca}, where a_{Ca} is the calcium activity, and C_{Ca} is the concentration of ultrafilterable calcium.
[4] Average from 10 to 12 hens ± SD. Differences among segments were highly significant ($P < 0.01$) for ultrafilterable Ca and Ca activity, and significant ($P < 0.05$) for the active fraction.

TABLE 4

Calcium concentration in the blood plasma of the laying fowl

Calcium fraction	Concentration
	mmoles/liter
Total calcium	5.5 ± 0.9 [1]
Ultrafilterable calcium	2.1 ± 0.5
Calcium activity [2]	1.1 ± 0.1
Active fraction of total calcium [3]	0.200
Active fraction of ultrafilterable Ca	0.525

[1] Average from 6 hens ± SD.
[2] Calcium activity was measured with an Orion calcium electrode.
[3] Active fraction = a_{Ca}/C_{Ca}, where a_{Ca} is the calcium activity, and C_{Ca} is the concentration of ultrafilterable calcium.

ported for mammal species and was mostly not diffusible, in agreement with Urist et al. (16). It is of interest that the fraction of active calcium in the plasma is close to the theoretical activity coefficient of 0.56, which indicates that most of the ultrafilterable calcium in this fluid is in ionic form.

Trial 3. This trial was designed to evaluate the effect of dietary calcium source, in a low calcium diet, on the activity and ultrafilterable concentration of calcium in the intestinal digesta. In addition to those measurements, the response of the ultrafilterable phosphate and intraluminal pH to the dietary treatments was evaluated.

To a basal diet containing 1.30% dicalcium phosphate, calcium was added from 3 sources: limestone (calcium carbonate), dicalcium phosphate and calcium sulfate (table 1). The concentration of calcium was maintained at about 1.3% in all diets.

Calcium activity and ultrafilterable calcium were similar in the duodenum and the jejunum except for the birds fed calcium sulfate, in which ultrafilterable calcium increased from the duodenum to the jejunum; they then markedly dropped in the ileum (fig. 1). Except for the duodenum, where no significant difference between the diets was found, the calcium activity was higher in the hens fed calcium sulfate than in the others ($P < 0.05$). There was no significant difference ($P > 0.05$) between the birds fed calcium carbonate and those fed dicalcium phosphate. In all intestinal segments the ultrafilterable calcium was significantly higher in the birds fed calcium sulfate ($P < 0.01$), with no such difference between the calcium carbonate and the dicalcium phosphate treatments.

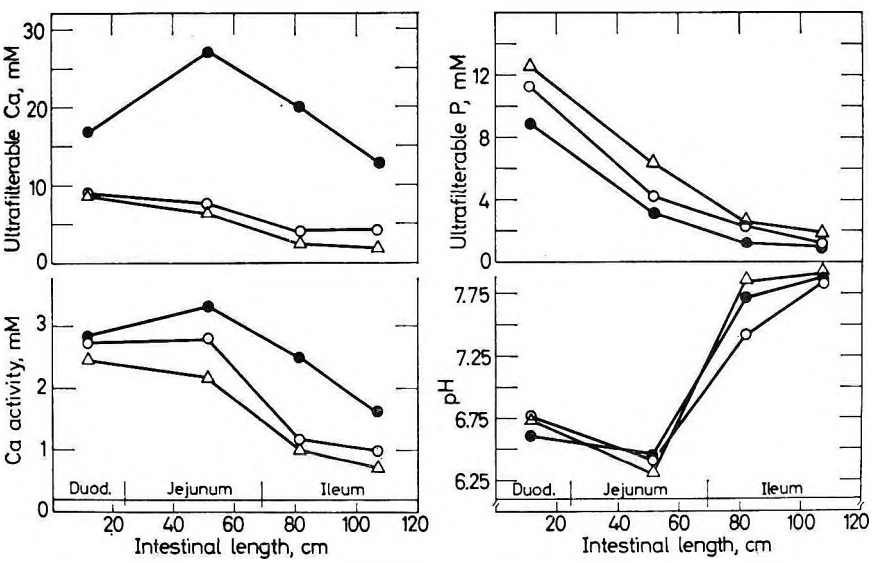

Fig. 1 Effect of dietary limestone (○), dicalcium phosphate (△), and calcium sulfate (●) on the calcium activity and concentration and phosphate concentration in intestinal ultrafiltrates, and on intraluminal pH. The average coefficient of variation was 36.1%, 51.3%, 38.1% and 3.9%, for calcium activity, calcium concentration, phosphate concentration and pH, respectively.

TABLE 5

Effect of various calcium supplements on the fraction of active calcium in the intestinal contents of laying hens [1]

Intestinal segment	Calcium supplement		
	Limestone	Dicalcium phosphate	Calcium sulfate
Duodenum	0.315	0.264	0.175
Jejunum	0.303	0.342	0.136
Ileum, upper	0.339	0.391	0.143
Ileum, lower	0.293	0.389	0.146

[1] Fraction of active calcium = calcium activity/ultrafilterable calcium concentration. The average coefficient of variation was 27.3%.

Neither ultrafilterable phosphate nor intraluminal pH was significantly ($P > 0.05$) influenced by the dietary treatments (fig. 1). However, ultrafilterable phosphate tended to be somewhat higher in the birds fed dicalcium phosphate, and lower in those fed calcium sulfate than in those fed calcium carbonate. The highest concentration of ultrafilterable phosphate appeared in the duodenum with a rapid decrease in concentration with the distance from the pylorus.

No consistent changes were noted in the fraction of active calcium along the intestine, with any of the dietary treatments (table 5). This fraction was significantly lower for the birds fed calcium sulfate ($P < 0.01$), but there was no significant difference between those fed calcium carbonate or dicalcium phosphate ($P > 0.05$).

DISCUSSION

Both ultrafilterable calcium and calcium activity were found to be considerably higher in the intestinal digesta than in blood plasma in laying hens under normal dietary conditions. This finding is in agreement with observations of Cramer (7) on the solubility of calcium in the intestine of the dog. As has been suggested for sheep intestine (8, 9) the solubility of calcium appears to be related to the intraluminal pH. This is apparent from the decrease in the ultrafilterable calcium in the ileum corresponding to an increase in the pH (table 2). It is of interest that except for the duodenum, only a small fraction of the total intestinal calcium was ultrafilterable.

In addition to the pH, the concentration and activity of calcium in the digesta appear to be markedly influenced by the dietary calcium level and source. Comparison of the observations on hens fed the high calcium diet (table 3) with those of others fed the low calcium diet with calcium carbonate, (fig. 1) shows that both calcium activity and ultrafilterable calcium were higher for the birds fed high calcium. Calcium sulfate, as compared with the other calcium supplements, promoted a higher concentration and activity of calcium in the ultrafiltrates. This effect is apparently not due to any change in intestinal pH. There was little difference in the solubility of calcium from the calcium carbonate and the dicalcium phosphate, although it tended to be somewhat lower in the latter as may be expected from the known reciprocal relationships between calcium and phosphorus solubilities.

The fraction of active calcium in the blood plasma was close to the theoretical activity coefficient based on the ionic strength, which indicates that the major portion of the ultrafilterable calcium in plasma is in ionic form. However, the fraction of active calcium in the intestinal ultrafiltrates was only about one-half of the theoretical activity coefficient, probably due to the presence of low molecular weight complexing agents which can pass through the dialysis membrane. The fraction of active calcium in birds fed the high calcium diet (table 3) decreased with the distance from the pylorus, most markedly from the jejunum to the ileum. Since the main decrease in the fraction of active calcium was associated with a decrease in both concentration and activity of calcium, it is possible that due to the increase in pH (table 2), less of the ionic calcium could stay in solution, thus increasing the relative portion of the complexed calcium.

The change in dietary calcium level did not markedly alter the fraction of active calcium (tables 3 and 5). In the posterior segments only, this fraction appears to be somewhat higher for the birds fed low calcium. However, the anionic counterpart of the calcium supplement modified this fraction considerably, as shown by the low values obtained with the birds fed calcium sulfate. This may be due to an excess of sulfate ions in the solution which lowers the activity of calcium, i.e., a high concentration of undissociated calcium sulfate.

It is tempting to explain the solubility of calcium in the intestinal contents on the basis of solubility products of the various calcium salts, since only a small fraction of the total intestinal calcium is in solution (table 2). However, due to the complexity of the system which includes several calcium salts as well as many other electrolytes and nonelectrolytes, it would be useless to speculate on this point.

The transmural PD is similar in sign and of the same order of magnitude as values reported for other species. Schoffeniels (5) and Clarkson (4) have compared the potential across the small and the large intestine in several animals. The colon always had a large potential, of the order of 20 to 40 mV, as compared with the small intestine, which was generally less than 10 mV. The present data on the laying fowl correspond well with this pattern. A single point of difference is that, in the rat, the lowest potential is observed in the lower ileum (4) whereas it occurs between the jejunum and ileum in the fowl. However, the observations with the rat were made on a preparation in vitro.

The electrochemical potential difference of calcium was calculated from the equation:

$$\frac{\tilde{\mu}_m - \tilde{\mu}_b}{F} = \frac{RT}{F} \ln \frac{a_m}{a_b} + Z(\psi_m - \psi_b)$$

where, a_m and a_b are the activities of calcium in the intestinal lumen and blood plasma, respectively; $(\psi_m - \psi_b)$ is the electrical potential difference (PD), T is the absolute temperature; R, F and Z are the gas constant, Faraday number and valence, respectively.

The rate of calcium absorption in the various intestinal segments was calculated from results of a previous study (1), in which yttrium-91 was mixed into the diet as a reference substance. From those results, the data from the hens fed a 3.56% calcium diet during egg shell formation were used. The rate of calcium absorption can be expressed as:

$$Ca_{abs} = {}^{91}Y_{intake}\, \Delta Ca/{}^{91}Y$$

where calcium absorption is expressed in μmoles/hour, ${}^{91}Y_{intake}$ in cpm/hour, and $\Delta Ca/{}^{91}Y$ is the change in the $Ca/{}^{91}Y$ ratio along the segment, obtained graphically from the plot of $Ca/{}^{91}Y$ ratio compared with the length of the intestine.

Comparison of the electrochemical potential difference and the absorption of calcium along the intestine is shown in table 6. The electrochemical potential difference was positive in the duodenum, jejunum and upper ileum, but negative in the lower ileum and colon. It is apparent that net absorption occurred in those segments where the potential difference of calcium was positive. On a qualitative basis, it thus appears that absorption occurs only under a favorable electrochemical potential difference for it. However, there is no

TABLE 6

Relationship between absorption and the electrochemical potential difference of calcium across the intestine of the laying fowl [1]

Intestinal segment	Ca/Y ratio		Ca absorption		ECPD [2]
	Avg	Δ			
	mg/cpm × 10^{-4}		*μmoles/hr*	*μmoles/hr/cm*	*mV*
Duodenum	10.3	6.0	1532	61.3	14.7
Jejunum, upper	7.0	2.4	612	27.9	15.0
Jejunum, lower	5.3	1.1	280	12.7	36.7
Ileum, upper	4.8	0.5	127	4.9	15.2
Ileum, lower	4.2	0.0	0	0.0	−13.3
Colon	4.7	0.0	0	0.0	−35.8

[1] The electrochemical potential difference was calculated from the equation

$$\frac{\tilde{\mu}_m - \tilde{\mu}_b}{F} = \frac{RT}{F} \ln \frac{a_m}{a_b} + Z(\psi_m - \psi_b)$$

where, a_m and a_b are the ion activities in the intestinal lumen and blood plasma, respectively; $(\psi_m - \psi_b)$ is the mucosa-blood PD; T is the absolute temperature; R, F and Z are the gas constant, Faraday number and valency, respectively. The rate of calcium absorption was calculated from previous results (1); for explanation, see text.

[2] ECPD = electrochemical potential difference.

correlation between the rate of calcium absorption and the magnitude of the electrochemical potential difference. Factors such as permeability of the mucosa and possibly active transport may also be responsible for part of the differences among the intestinal segments in their rate of calcium absorption. Such differences have been reported for the rat intestine in vitro (3).

The driving forces shown in table 6 refer only to the ionic calcium. It was concluded earlier in this discussion that a significant portion of the soluble calcium in the gut contents was in the form of small ultrafilterable complexes. There is no evidence on hand that indicates the chemical nature of the complexing substance, but one report (17), based on studies with the rat, implicated phosphate. Preliminary results in our laboratory with in vitro preparations suggest that absorption of complexed calcium may occur.[2] Until the chemistry and physiology of the complexing agents in the gut contents of the laying fowl are better understood, it is fruitless to speculate on the nature of the driving forces operating on the calcium complexes.

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Effect of Various Energy Sources upon Plasma Free Amino Acids in Sheep [1]

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ABSTRACT Intra-arterial infusion of energy sources were made in view of the possibility that energy might induce short-term changes in the plasma free amino acid (PFAA) concentration which would in turn reflect the limiting amino acid in ruminants. Changes in the PFAA concentrations after energy infusions are expressed as plasma amino acid indexes (PAAI) and are defined as the (PFAA concentration after energy infusion divided by the PFAA concentration pre-energy infusion) × 100. In the first of three 4 × 4 Latin-square design experiments intra-arterial infusion of glucose depressed the PFAA below the PFAA concentration after saline (control) infusions at both 24 and 6 hours post-feeding. Furthermore, depressions in the essential amino acids after glucose infusions were greater than the decreases in the nonessential amino acids at both times. In the second experiment isocaloric infusions of glucose, propionate, acetate and butyrate resulted in average plasma essential amino acid indices of 59, 66, 90 and 85, respectively. In the last experiment the energy infusions were glucose, acetate, half glucose plus half acetate isocaloric with the first treatments and half glucose plus half acetate at twice the caloric level of the other treatments. All infusions in experiments 2 and 3 were at 24 hours post-feeding. Correlations of the essential amino acid reduction patterns observed after infusion of glucose 24 hours post-feeding with the relative essential amino acid composition of lamb for the 3 experiments were 0.89, 0.72 and 0.94, respectively. The plasma amino acid indexes indicated isoleucine as the limiting amino acid (lowest index) after the following infusions: glucose 24 hours post-feeding (all experiments), propionate, and both half glucose-half acetate treatments in experiment 3.

In a previous paper from this laboratory (1) the use of modified plasma amino acid ratios was suggested as a possible way by which the limiting amino acid in ruminants could be determined. In that study both a high energy diet and a starch-glucose mixture infused into the rumen resulted in depressions in the plasma free amino acid concentrations below the pre-feeding or pre-energy infusion levels, respectively. Since the ruminant normally uses short-chain volatile fatty acids as well as limited amounts of glucose, the experiments reported here were designed to monitor the effects of various intra-arterial energy infusions upon the plasma free amino acid concentrations in sheep and at the same time to further evaluate the use of the modified ratios in delineating the limiting amino acids in ruminant animals.

METHODS

The treatments used in three 4 × 4 Latin-square design experiments are shown in table 1. The sheep used in all 3 experiments were 2.5-year-old wethers and were fed once a day at 6%, 7% and 6% of the metabolic body ($BW^{0.75}$) for the 3 experiments, respectively. The ration was identical in composition to ration B previously reported (1).

An initial 21-day-ration adjustment period was followed by treatments (infusion of energy into carotid artery) every seventh day. The energy infusions, which took approximately 1.5 minutes to complete, were neutralized with NaOH, and prepared such that all treatments to one sheep were of equal volume as well as isocaloric with the glucose treatment except for the glucose plus acetate treatment (exp. 3) which was double the caloric level of the other treatments. The amount of glucose infused was 0.05% of $BW^{0.75}$. In experiment 1, two of the infusions, and in experiments 2 and 3, all the energy infusions were made 24 hours after the last feeding. The other 2 infusions in experi-

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TABLE 1

Experimental design and animal data

Treatment [1]		
Exp. 1 [2]	Exp. 2 [3]	Exp. 3 [3]
a. Glucose 24 hr post-feeding	a. Glucose	a. Glucose
b. Saline 24 hr post-feeding	b. Propionate	b. Acetate
c. Glucose 6 hr post-feeding	c. Butyrate	c. ½ (glucose + acetate)
d. Saline 6 hr post-feeding	d. Acetate	d. Glucose + acetate [4]

Animal data			
Sheep	$BW^{0.75}$	Calories infused [5,6]	Exp. no.
	kg	*kcal*	
10	17.5	32.7	1, 2, 3
11	19.0	35.5	1, 2
12	17.0	31.7	1
13	18.5	34.5	1, 2, 3
17	20.5	38.3	3
18	20.0	37.4	2
19	21.3	39.8	3

[1] Treatments were at 24-hours post-feeding unless otherwise indicated.
[2] Samples were collected at zero, 1, 3 and 5.5 hours after energy infusion.
[3] Samples were collected at zero and 1 hour after energy infusion.
[4] This treatment was twice the caloric level of the other treatments.
[5] Treatments were isocaloric with the glucose treatment which was infused by weight at 0.05% of the metabolic body weight ($BW^{0.75}$).
[6] Infusions were over a period of 90 seconds and the volume depended upon body weight (volumes ranged between 25 to 35 cm^3 per infusion).

ment 1 were made 6 hours after feeding. Blood samples were collected immediately before the energy infusions, as well as one hour after the infusions in all 3 experiments. Additional post-treatment blood samples were taken in experiment 1; these were at 3 and 5.5 hours post-infusion. The blood samples were prepared and analyzed as previously described (1) with the exception that sulfosalicylic acid filtrates were used for plasma free amino acid determinations. Norleucine was used as an internal standard.

The ratios developed by dividing post-treatment plasma amino acid concentrations by pre-treatment plasma amino acid concentrations × 100 are referred to as plasma amino acid indexes (PAAI). Since PAAI's facilitate interpretation of the data and allow comparisons to be made between amino acids, the PAAI's and not the post-treatment plasma amino acid concentrations appear in tables 2, 3, and 4. Significant differences found after covariance analysis of the post-treatment plasma amino acid concentrations with respect to the pre-treatment plasma amino acid concentrations are indicated by an asterisk after the amino acid. Furthermore differences between treatment means separated according to Duncan's multiple range test (3) are indicated by like or unlike letters after the indexes (like letters indicate like means). The PAAI's were statistically analyzed by analysis of variance; although there were slight differences between the two methods of analysis the differences indicated in this paper are based only on the covariance analysis.

RESULTS

Shown in table 2 are the pre-treatment plasma essential amino acid concentrations and the PAAI's derived from the pre-infusion and one-hour post-infusion plasma amino acid concentrations, for experiment 1. Significant differences ($P < 0.05$) between treatments were shown for valine and leucine. Thus glucose infusion 24 hours post-feeding resulted in depressions in the plasma concentration of both valine and leucine below the concentration after saline infusion 24 hours post-feeding. This is reflected by the PAAI's, for example, the values for valine were 73 and 95 after the glucose and saline infusions, respectively. Even though the mean index for isoleucine one hour after the 24-hour post-feeding glucose infusion was less than that for leucine and valine, the plasma amino acid

TABLE 2

Mean [1] pre-treatment plasma amino acid concentration and one-hour post-treatment indexes [2] (exp. 1)

Amino acid	Pre-infusion concn				1-hr post-treatment indexes			
	24-hr post-feeding		6-hr post-feeding		24-hr post-feeding		6-hr post-feeding	
	Glucose	Saline	Glucose	Saline	Glucose	Saline	Glucose	Saline
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*				
Thr	2.05 ± 0.28 [3]	1.80 ± 0.28	1.51 ± 0.57	1.42 ± 0.14	83 ± 8	98 ± 14	83 ± 28	97 ± 8
* Val [4]	2.20 ± 0.43	2.01 ± 0.43	1.61 ± 0.24	1.72 ± 0.30	73 ± 4 [a 5]	95 ± 11 [b]	94 ± 7 [b]	100 ± 10 [b]
Met	0.36 ± 0.05	0.35 ± 0.06	0.24 ± 0.03	0.24 ± 0.03	76 ± 9	77 ± 9 [6]	*81 ± 4* [6]	93 ± 6
Ile	1.13 ± 0.30	0.95 ± 0.19	0.91 ± 0.15	0.80 ± 0.09	63 ± 8 [6]	89 ± 12	83 ± 2	114 ± 30
* Leu	1.76 ± 0.27	1.71 ± 0.35	1.34 ± 0.24	1.24 ± 0.21	69 ± 12 [a]	88 ± 10 [bc]	83 ± 3 [ab]	107 ± 3 [c]
Tyr	1.23 ± 0.18	1.20 ± 0.39	1.03 ± 0.27	1.11 ± 0.35	83 ± 10	90 ± 18	88 ± 6	*90 ± 15* [6]
Phe	0.90 ± 0.06	0.88 ± 0.22	0.70 ± 0.12	0.70 ± 0.10	85 ± 14	89 ± 4	91 ± 12	101 ± 5
Lys	2.16 ± 0.63	1.70 ± 0.62	1.35 ± 0.19	1.19 ± 0.29	72 ± 16	80 ± 10	83 ± 17	93 ± 6
His	1.36 ± 0.69	1.05 ± 0.38	1.19 ± 0.46	1.07 ± 0.15	97 ± 13	100 ± 30	94 ± 14	112 ± 16
EAA [7]	13.16	11.65	9.88	9.49	78	90	87	101
NEAA [8]	34.78	29.92	27.58	27.55	95	95	93	98

[1] Mean of 4 sheep.
[2] Indexes are the post-treatment concentrations divided by the pre-treatment concentrations × 100.
[3] SD.
[4] Amino acid which showed ($P < 0.05$) differences due to treatment as indicated by an asterisk.
[5] Unlike letters indicate means which are different ($P < 0.05$) determined by Duncan's multiple range test (3).
[6] Index values in italics indicate the lowest index in each treatment or the limiting essential amino acid.
[7] Either total essential amino acid concentration or average essential amino acid index.
[8] Either total nonessential amino acid concentration or average nonessential amino acid index.

TABLE 3

Mean [1] pre-treatment plasma amino acid concentration and one-hour post-treatment indexes [2] (exp. 2)

Amino acid	Pre-infusion concn				1-hr post-treatment indexes			
	Glucose	Propionate	Butyrate	Acetate	Glucose	Propionate	Butyrate	Acetate
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*				
Thr	1.95 ± 0.47 [3]	1.75 ± 0.64	1.73 ± 1.14	2.21 ± 0.55	63 ± 16	68 ± 16	92 ± 42	*73 ± 21* [6]
* Val [4]	2.21 ± 0.69	1.89 ± 0.39	1.65 ± 0.48	2.29 ± 0.50	65 ± 15 [a 5]	71 ± 15 [a]	93 ± 10 [ab]	91 ± 12 [b]
* Met	0.37 ± 0.06	0.33 ± 0.06	0.26 ± 0.10	0.35 ± 0.04	56 ± 12 [a]	61 ± 13 [a]	*73 ± 7* [a 6]	99 ± 22 [b]
* Ile	1.00 ± 0.15	0.90 ± 0.18	0.70 ± 0.24	1.00 ± 0.07	*49 ± 4* [a 6]	*53 ± 16* [a 6]	76 ± 6 [a]	98 ± 28 [b]
* Leu	1.75 ± 0.39	1.57 ± 0.32	1.27 ± 0.59	1.76 ± 0.13	53 ± 17	60 ± 14 [ab]	85 ± 8 [b]	89 ± 11 [c]
* Tyr	1.47 ± 0.49	1.18 ± 0.21	1.12 ± 0.35	1.38 ± 0.34	63 ± 11 [a]	74 ± 9 [a]	92 ± 15 [a]	101 ± 23 [b]
* Phe	1.11 ± 0.36	0.89 ± 0.18	0.89 ± 0.36	1.11 ± 0.08	64 ± 11 [a]	75 ± 12 [a]	89 ± 17 [a]	91 ± 12 [b]
Lys	1.81 ± 0.36	1.91 ± 0.63	1.54 ± 0.43	2.14 ± 1.34	60 ± 12	57 ± 14	75 ± 16	85 ± 20
His	1.11 ± 0.24	1.04 ± 0.25	0.84 ± 0.29	1.09 ± 0.29	61 ± 17	76 ± 12	88 ± 13	84 ± 10
EAA [7]	12.78	11.46	10.00	13.33	59	66	85	90
NEAA [8]	33.84	29.90	27.41	32.35	70	72	93	88

[1] Mean of 4 sheep.
[2] Indexes are the post-treatment concentration divided by the pre-treatment concentration × 100.
[3] Average SD.
[4] Asterisk indicates those amino acids which were different due to treatment ($P < 0.05$).
[5] Unlike letters indicate means which are different ($P < 0.05$), determined by Duncan's multiple range test (3).
[6] Index values in italics indicate the lowest index in each treatment or the limiting essential amino acid.
[7] Either total essential amino acid concentrations or average essential amino acid index.
[8] Either total nonessential amino acid concentrations or average nonessential amino acid index.

TABLE 4

Mean [1] pre-treatment plasma amino acid concentration and one-hour post-treatment indexes [2] (exp. 3)

Amino acid	Pre-infusion concn				1-hr post-treatment indexes			
	Glucose	Acetate	Glucose + acetate/2	Glucose + acetate	Glucose	Acetate	Glucose + acetate/2	Glucose + acetate
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*				
Thr	2.08 ± 1.39 [3]	2.11 ± 0.42	1.36 ± 0.62	1.47 ± 0.73	78 ± 13	76 ± 12	89 ± 7	95 ± 44
Val	2.43 ± 1.24	2.34 ± 0.31	1.61 ± 0.56	2.33 ± 0.85	78 ± 14	83 ± 19	95 ± 12	87 ± 17
Met	0.38 ± 0.19	0.38 ± 0.07	0.25 ± 0.10	0.31 ± 0.12	75 ± 11	80 ± 20	104 ± 21	84 ± 26
Ile	1.11 ± 0.53	1.02 ± 0.22	0.92 ± 0.27	1.16 ± 0.37	60 ± 10 [4]	72 ± 28	88 ± 14 [4]	70 ± 29 [4]
Leu	1.95 ± 0.83	1.66 ± 0.08	1.15 ± 0.24	1.88 ± 0.94	66 ± 5	86 ± 21	92 ± 16	76 ± 30
Tyr	1.07 ± 0.48	0.90 ± 0.08	0.69 ± 0.17	0.93 ± 0.39	75 ± 12	88 ± 19	98 ± 18	76 ± 8
Phe	1.39 ± 0.76	1.33 ± 0.14	0.94 ± 0.22	1.30 ± 0.42	76 ± 12	85 ± 19	93 ± 14	84 ± 20
Lys	2.18 ± 1.15	2.37 ± 0.54	1.72 ± 0.64	1.86 ± 0.94	77 ± 14	71 ± 17 [4]	93 ± 16	89 ± 40
His	1.19 ± 0.22	1.65 ± 0.59	0.96 ± 0.20	1.10 ± 0.33	80 ± 15	74 ± 11	91 ± 7	93 ± 20
EAA [5]	13.78	13.76	9.60	12.34	74	79	94	84
NEAA [6]	35.95	39.30	30.77	33.16	83	76	91	82

[1] Mean of 4 sheep.
[2] Indexes are the post-treatment concentration divided by the pre-treatment concentration × 100.
[3] SD.
[4] Index values in italics indicate the lowest index or limiting amino acid in each treatment.
[5] Either total essential amino acid concentrations or average essential amino acid index
[6] Either total nonessential amino acid concentrations or average nonessential amino acid index.

depression was not significant and variability arising from one animal apparently accounted for this nonsignificance. However, the depression of the plasma isoleucine 3 hours after the infusion was significant.

In general, comparison of the glucose and saline treatments shows larger depressions in the plasma essential amino acid indexes after glucose treatment at both infusion times. After the 24-hour post-feeding infusion of glucose, the average plasma essential amino acid index was 78, whereas it was 90 after the infusion of saline. At 6 hours post-feeding the average plasma essential amino acid index was 87 after glucose infusion and 101 after saline infusion. These differences between the glucose and saline treatments were not apparent for the nonessential plasma free amino acid indexes.

Both the average essential and nonessential PAAI for all 3 post-infusion sampling times for experiment 1 are shown in figure 1. In general, the trends noted at the one-

Fig. 1 Average essential and non-essential plasma amino acid indexes 1, 3 and 5.5 hours after glucose and saline infusions 24 and 6 hours post-feeding.

hour post-infusion time for both 24-hour post-feeding and 6-hour post-feeding were also present at the two later collection times.

The mean pre-infusion plasma essential amino acid concentrations and the essential PAAI's for the treatments of experiment 2 are shown in table 3. Significant differences between treatments are indicated as described for experiment 1. The following essential amino acids showed significant differences due to treatments: valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine. For all the essential amino acids, the PAAI's obtained after both glucose and propionate treatments were similar to each other as well as lower than the indexes after acetate treatment. This is reflected by the average essential amino acid indexes (EAAI's) after glucose and propionate of 59 and 66, respectively, whereas the average index after acetate treatment was 90. Furthermore, acetate was the only treatment which did not depress the average essential amino acid index below the average nonessential amino acid index (NEAAI).

The mean pre-infusion plasma essential amino acid concentrations and the indexes for the treatments in experiment 3 are shown in table 4. No significant differences between treatments were found with covariance analysis. However, all 3 treatments in which acetate was infused agree with the acetate treatment in experiment 2 in that the average essential amino acid indexes were not depressed below the average nonessential amino acid indexes.

The relative reduction in each of the plasma essential amino acids, expressed as a percentage of the total plasma essential amino acid reduction following the energy infusions in all three experiments are shown in table 5. Comparisons of these plasma essential amino acid depression patterns after each treatment with the essential amino acid composition of lamb or mutton were made and these correlations as well as the percentage distribution of the essential amino acid composition of lamb or mutton also appear in table 5. Glucose infusion 24 hours post-feeding resulted in depression patterns which consistently gave high correlations with the composition of lamb or mutton, 0.89, 0.72

TABLE 5

Essential amino acid composition of lamb or mutton,[1] plasma essential amino acid reduction pattern [2] and the correlations of the composition with the reduction pattern for the various energy infusions

EAA	Mutton or lamb [3]	Exp. 1		Exp. 2				Exp. 3			
		Glu, 24-hr	Glu, 6-hr	Glu	Propionate	Butyrate	Acetate	Glu	Ac	Glu + Ac/2	Glu + Ac
	%	%	%	%	%	%	%	%	%	%	%
Thr	10.3	10.1	24.3	15.1	13.8	25.7	36.7	11.5	15.9	17.4	0.0
Val	11.7	19.3	7.2	14.9	13.3	8.0	14.0	15.3	12.4	10.2	16.4
Met	5.4	3.0	3.2	3.1	2.9	1.8	0.0	2.6	2.5	1.1	1.9
Ile	11.6	14.3	10.7	9.8	10.0	4.4	1.3	11.5	9.6	13.6	18.5
Leu	17.4	18.0	16.5	15.8	14.7	8.8	14.0	16.3	7.4	12.4	24.9
Tyr	7.8	6.7	9.3	10.6	7.1	10.6	0.0	9.1	6.8	10.2	9.1
Phe	9.1	4.7	3.6	7.5	12.4	18.1	7.3	7.2	3.7	3.4	10.5
Lys	21.1	21.3	17.2	14.5	20.2	17.7	14.7	20.6	30.3	20.4	14.7
His	6.3	2.0	7.9	8.7	5.7	4.9	12.0	6.0	12.1	11.4	4.0
Total	100.7	100.0	99.9	100.0	100.1	100.0	100.1	100.1	99.9	100.2	100.0
Correlation	—	0.89	0.57	0.72	0.90	0.33	0.29	0.94	0.69	0.68	0.68

[1] Home Economic Research Report no. 4, USDA 1957 Amino acid content of food.
[2] Individual plasma EAA reductions expressed as a percentage of the total plasma EAA reduction after the various energy infusions.
[3] Expressed as a percentage of the total EAA composition of lamb or mutton.

and 0.94 for the 3 experiments, respectively.

DISCUSSION

Munro and Thompson (4), Crofford et al. (5) and Swendseid et al. (6) have reported decreases in the concentration of the essential amino acids after ingestion of glucose in human subjects. Furthermore, they suggested that protein synthesis was taking place because the decrease in the essential amino acids closely reflected either the amino acid requirements of man or the amino acid composition of mammalian tissue. Recently Rao and McLaughlan (7) reported data which support these findings. The results of experiment 1, namely the depression in the plasma essential amino acids by glucose infusion below the level observed after saline infusion and in addition the high correlations obtained between the plasma essential amino acid composition of lamb, agree with these results and also support previous results indicating that the addition of readily available energy to the rumen will decrease the plasma free amino acid concentrations in ruminants (1).

Even though either cellular uptake of amino acids or protein synthesis or both is suggested by the present work, it is realized that other interpretations for depressions in the plasma free amino acid concentrations can be made since numerous factors affecting plasma amino acid concentrations are well-documented (8, 9). In fact an effect of feeding is shown by the present data for the lower correlation (0.57) between the depression pattern and mutton composition after the glucose infusion 6 hours post-feeding may have been due to the additional supply of essential amino acids available for absorption from the gut. In effect, this may provide quantities of amino acids for replenishing the plasma pool at the time of the 6-hour post-feeding energy infusion, and presumably the composition of this amino acid supply would not be the same as the depression pattern resulting from the glucose infusion; hence the lower correlation value. The effect of feeding can also be observed by comparison of the indexes after saline infusion 24 hours post-feeding with the indexes after saline infusion 6 hours post-feeding; as shown in figure 1, the values are shifted upward. Likewise, these same differences exist between the indexes after the infusion of glucose 24 and 6 hours post-feeding. These differences are considered to be related to factors influencing plasma amino acid concentrations such as passage to the lower gut, digestion, amino acid absorption and energy supply.

In two studies Swendseid et al. (10, 11) showed reductions in the ratio of plasma essential to nonessential amino acids (EN ratio) with reductions in the level of dietary protein. The decreased EN ratios were a net result of a decrease in essential amino acids and in most cases an increase in the nonessential amino acids, which was thought to be due to continued synthesis of nonessential amino acids despite restricted protein synthesis. In each of the present experiments, glucose caused greater average reductions in the essential amino acids than in the nonessential amino acids and this may also be attributable to synthesis of nonessential amino acids.

Experiment 2 was conducted to determine whether the various energy sources available to the ruminant have different effects upon the plasma free amino acids. The results indicate that both the relative degree of depression and the depression pattern of the essential amino acids are dependent upon the energy source infused. Thus the average treatment essential amino acid indexes (table 3) and the correlations of plasma depression patterns with the essential amino acid composition of mutton (table 5) varied markedly between energy treatments. Correlations after glucose and propionate infusions were high, 0.72 and 0.90, respectively, whereas correlations after butyrate and acetate infusions were only 0.33 and 0.29, respectively. These correlation values suggest that more short-term protein synthesis or cellular uptake of amino acids resulted from either glucose or propionate infusions than from butyrate or acetate infusions. Relatively little protein synthesis following acetate infusion is also suggested by the fact that the average EAAI's were not depressed below the average NEAAI's since this suggests that amino acids were not removed in quantity from the plasma pool. These

results may be explained in part by the results of Mayfield et al. (12) who showed that the decreasing order in the relative rate of $^{14}CO_2$ production from acetate-1-^{14}C by ruminant tissues was adipose, kidney, muscle, heart, lung, brain and liver. Thus it is conceivable that glucose and acetate were metabolized predominantly by different tissue and that cellular uptake or protein synthesis per unit of energy in these tissues was not equal.

The above reasoning may also explain the discrepancy between the correlation values obtained after the acetate infusions (0.33 and 0.69) for the dietary level in experiments 2 and 3 was 7% and 6% $BW^{0.75}$, respectively, and this may have influenced the tissues metabolizing the major portion of the energy infused.

The validity of the plasma amino acid index method for the prediction of the limiting amino acid depends upon whether or not the composition of the amino acids removed from the plasma by either cellular uptake or protein synthesis following energy infusion is proportional to the average daily amino acid requirement of the animal. When protein synthesis occurs, the essential amino acid index which is lowest would indicate the limiting essential amino acid since this value represents the greatest relative decrease with respect to the preinfusion concentration of each essential amino acid.

The limiting essential amino acids, that is, the essential amino acid showing the lowest index for each treatment, are designated in tables 2, 3, and 4 by the italicized index. The PAAI method was consistent since glucose infusions in all three experiments showed isoleucine as the limiting essential amino acid.

While the validity of this method in its ability to determine the limiting essential amino acid is far from being proven, it is considered that this is potentially the simplest and least complicated method for determining the limiting amino acid in ruminants. In part, the principle of the technique is similar to that of McLaughlan (13), Longenecker and Hause (14) and Smith and Scott (15), for it is based upon the use of reference concentrations of amino acids, in this case the pre-infusion concentrations. It seems necessary to emphasize this relationship because in most instances a simple plasma free amino acid concentration is of little value for nutritional interpretation purposes.

ACKNOWLEDGMENT

Appreciation is extended to Dr. F. R. Allaire for statistical assistance.

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Invitation for Nominations for 1969 American Institute of Nutrition Awards

Nominations are requested for the 1969 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) *A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1968,* to be considered for the 1969 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made during the banquet at the annual meeting.

1969 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper
1966 – R. T. Holman
1967 – R. H. Barnes
1968 – C. L. Comar

NOMINATING COMMITTEE:

K. E. HARSHEARGER, *Chairman*
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Send nominations to:

K. E. HARSHEARGER
Department of Dairy Science
University of Illinois
Urbana, Illinois 61801

1969 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.
1965 – D. M. Hegsted
1966 – H. H. Mitchell
1967 – Samuel Lepkovsky
1968 – C. H. Hill

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Send nominations to:

L. E. HOLT, JR.
Professor of Pediatrics
New York University School of Medicine
550 First Avenue
New York, N. Y. 10016

1969 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri
1966 – M. Daniel Lane
1967 – W. N. Pearson
1968 – H. F. DeLuca

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Department of Biochemistry
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St. Paul, Minnesota 55101

1969 Conrad A. Elvehjem Award for Public Service in Nutrition

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

Former recipients of this award are:

1966 – C. Glen King
1967 – J. B. Youmans
1968 – W. H. Sebrell, Jr.

NOMINATING COMMITTEE:
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Send nominations to:

R. E. SHANK
Department of Preventive Medicine
Washington University School of Medicine
4550 Scott Avenue
St. Louis, Missouri 63110

Invitation for Nominations for 1969 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

Fellows Committee:

RICHARD M. FORBES, *Chairman*
T. H. JUKES
L. A. MAYNARD
A. E. MORRISON

Send nominations to:

RICHARD M. FORBES
124 Animal Sciences Laboratory
University of Illinois
Urbana, Illinois 61801

The following persons have been elected previously as Fellows of the Society:

Georgian Adams (1967)
Herman J. Almquist (1968)
J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Earle W. Crampton (1967)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
L. Emmett Holt, Jr. (1967)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Max Kleiber (1966)
Samuel Lepkovsky (1966)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Paul H. Phillips (1968)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
W. H. Sebrell, Jr. (1968)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

R. W. ENGEL, *Chairman*
L. C. NORRIS
A. E. SCHAEFER

Send nominations to:

R. W. ENGEL
Associate Dean for Research
Virginia Polytechnic Institute
Blacksburg, Virginia 24061

The following persons have been elected previously as Honorary Members of the Society:

Kunitaro Arimoto
W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Dame Harriette Chick
F. W. A. Clements
Hans D. Cremer
Sir David P. Cuthbertson
Herbert M. Evans
Karl Guggenheim
Joachim Kühnau
Joseph Masek
Toshio Oiso
H. A. P. C. Oomen
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
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B. S. Platt
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Emile F. Terroine
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