Absorption of Amino Acids from the Small Intestine of Domestic Fowl

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ABSTRACT A series of experiments was carried out to examine some basic aspects of the in vivo absorption of L-amino acids in the chicken. When an equimolar mixture of 18 L-amino acids was injected into tied-off segments of bird intestine, it was observed that the absorption rate of amino acids was not dependent on their molecular weight; but amino acids with larger nonpolar side chains, such as methionine, isoleucine, valine, leucine, tryptophan and phenylalanine, were absorbed rapidly, whereas glycine and amino acids with polar side chains, such as glutamic acid, aspartic acid and arginine, were absorbed slowly. Amino acid absorption from the intestinal loop in situ was measured using a circulation unit when each of 2 L-amino acids was administered singly or in combination. The results showed that competitive inhibitory effects appeared in amino acid absorption. The absorption of leucine or phenylalanine was inhibited by methionine, but the inhibitory effect of leucine or phenylalanine on methionine absorption was not so great. When methionine was administered with glutamic acid, the absorption of glutamic acid was strongly inhibited, whereas the absorption of methionine was accelerated slightly. It was also observed that glutamic acid increased water absorption.

Early studies on the intestinal absorption of amino acids showed that 2 processes, diffusion and active absorption, were involved. Höber and Höber (1) observed that the absorption of glycine, alanine and valine was not proportional to the amino acid concentration in the gut, and that these amino acids were absorbed more rapidly than polyhydric alcohol of comparable molecular volume. From these observations, they concluded that active absorption was involved. On the other hand, comparing the rate of disappearance of several L- and DL-amino acids from the gut, Chase and Lewis (2) noted no difference in absorption between L-isomers and racemic mixtures. Bolton and Wright (3) observed a similar phenomenon, whereas Kratzer (4) showed that the rate of disappearance of amino acids from the gut was inversely proportional to the molecular volume of the amino acids. From these results, diffusion of amino acids from the intestinal wall also appears to be an important mechanism for amino acid absorption. Schofield and Lewis (5), Gibson and Wiseman (6), Wiseman (7) and Matthews and Smyth (8, 9) reported that L-amino acids were absorbed at a higher rate than the corresponding D-isomers.

When individual amino acids are introduced into the intestinal lumen separately, each amino acid is absorbed at a characteristic rate. However, these absorption rates vary with the presence of other amino acids, and this fact indicates that amino acids compete with each other for their absorption (10, 11).

Experiments using a single amino acid will give an indication of the basic mechanisms for amino acid absorption; however, it is also necessary to clarify the interaction of amino acids for their absorption when a mixture of amino acids is introduced into the intestinal lumen.

Although many studies have been made on rats, hamsters, dogs, cats and rabbits, there have been few reports on domestic fowls. The present experiments were carrid out to establish the absorption pattern of individual amino acids from an equimolar mixture of 18 L-amino acids in the small intestine of domestic fowls in situ, and to determine the absorption rate of each amino acid in situ using a circulation unit when one or two amino acids were administered equimolarly.

EXPERIMENTAL

Experiment 1 was undertaken to establish the pattern of amino acid absorption from the small intestine of domestic fowls

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in situ when an equimolar mixture of 18 L-amino acids was injected.

The procedure used was a modification of the technique established by Gibson and Wiseman (6). The birds were first anesthetized by an intravenous injection of sodium pentobarbital at a level of 25 mg/kg of body weight. The small intestine was exposed by making an incision and 10 ml of Krebs-Ringer phosphate buffer (pH 7.4) were injected into the intestinal lumen to be tested, and then forced manually toward the lower ileum so as to remove impurities in the lumen. The proximal and distal points of the intestine, each 10 cm from Meckel's diverticulum (the remnant of the attachment of the yolk stalk), were ligated, and 5 ml of solution with equimolar concentration of 18 L-amino acids (2.5 μmoles/ml each) were injected into the lumen. The intestine was returned to the abdominal cavity and the incision was clamped with hemostats.

Ten minutes after injection of the amino acid solution, the abdominal cavity was reopened and the sac of intestine was removed. The contents of the intestine were collected and the sac was washed thoroughly with distilled water. The collected intestinal contents and washings were then mixed and filtered. The filtrate was freeze-dried and dissolved in sodium citrate buffer (pH 2.2). Amino acids remaining in the solution were determined chromatographically by means of the Beckman Model 120B amino acid analyzer.

Experiment 2 was undertaken to determine the absorption rate of individual L-amino acids, such as methionine, leucine, phenylalanine, glycine and glutamic acid, when they were administered singly or in combination. The pairs of amino acid used were, methionine and leucine, methionine and phenylalanine, and methionine and glutamic acid. The in situ circulation unit developed by Fisher and Parsons (12) was modified slightly and used in the present experiment. Air was bubbled from the gas injector at a constant rate by regulating the compression valves so as to circulate water at a rate of 100 ml/minute. Two hundred milliliters of Krebs-Ringer phosphate buffer which contained one amino acid (10 μmoles/ml) or 2 amino acids (10 μmoles/ml, each) were introduced into the reservoir, and maintained at 42° by circulating warm water through the jackets.

The abdomen of the bird was opened by the same procedure described in experiment 1. The proximal and distal points of the intestine, 20 cm and 10 cm, respectively, from Meckel's diverticulum, were ligated and cut off carefully so as not to injure blood vessels. Impurities in the loop were washed out thoroughly with Krebs-Ringer phosphate buffer maintained at 42°, both ends of the intestinal loop with intact blood circulation were connected to the circulation unit, and the loop was then perfused. Ten-milliliter samples of the circulating fluid were taken by pipette from the reservoir at intervals of 15 minutes for an hour. During the circulation, the height of the circulating fluid was kept constant by elevating the reservoir. The intestinal loop was covered with gauze kept moist by warm Krebs-Ringer phosphate buffer.

The micro-Kjeldahl method described by Miller and Houghton (13) was used to determine total nitrogen. From a preliminary experiment, it was confirmed that little endogenous nitrogen was secreted into the intestinal loop during an hour's circulation; therefore, the amount of total nitrogen was considered to represent the amount of nitrogen of the amino acid remaining in the circulating fluid at a definite time.

When 2 amino acids were given simultaneously, it was necessary to determine each of them separately. If one of them was determined by a specific method, the other could be calculated by subtracting the nitrogen of the specifically determined amino acid from the total nitrogen determined by the micro-Kjeldahl method. In this experiment L-methionine was determined by a specific method described by Csonka and Denton (14).

In each experiment, Single Comb White Leghorn cockerels weighing 1.5 to 1.9 kg were used, and were fasted for 12 hours prior to the experiment.

RESULTS

The absorption pattern of amino acids from an equimolar mixture of 18 L-amino acids in the small intestine (exp. 1) is

shown in table 1. The amino acids with larger nonpolar side chains, such as methionine, isoleucine, valine, leucine, tryptophan and phenylalanine, belong to a group of rapidly absorbed amino acids, whereas glycine and the amino acids with polar side chains, such as glutamic acid, aspartic acid and arginine, belong to a group of slowly absorbed amino acids.

The characteristic absorption rates of L-amino acids and water from the circulating fluid containing a single amino acid (exp. 2) are shown in figure 1. The results show that the order of the absorption rate of amino acids is similar to that in experiment 1; i.e., methionine was absorbed most rapidly, followed by leucine, phenylalanine and glycine, with glutamic acid being the most slowly absorbed.

Table 2 shows the competition existing between various pairs of L-amino acids in their absorption process. When methionine was present with leucine or phenylalanine in the circulating fluid, the absorption rates of leucine and phenylalanine were 237.9 and 231.5 μmoles, respectively, per gram of dried intestine during 60 minutes, although the absorption rates of these amino acids were 322.5 and 310.3 μmoles, respectively, when each amino acid was administered separately. In contrast, changes

TABLE 1

Absorption pattern of individual amino acids from an equimolar mixture of 18 L-amino acids (12.5 μmoles/bird, each) in the small intestine during 10 minutes.

Amino acid	Absorbed
	%
Methionine	89.6 ± 3.3 [1]
Isoleucine	86.7 ± 4.2
Valine	86.0 ± 3.6
Leucine	84.9 ± 4.1
Tryptophan	83.3 ± 4.4
Phenylalanine	82.9 ± 4.2
Histidine	79.8 ± 4.3
Lysine	79.6 ± 4.9
Alanine	78.7 ± 5.1
Serine	78.5 ± 4.9
Threonine	78.4 ± 5.7
Tyrosine	78.2 ± 4.8
Cystine	77.7 ± 5.7
Proline	77.7 ± 6.4
Arginine	75.3 ± 6.1
Glycine	73.5 ± 8.1
Aspartic acid	69.2 ± 8.5
Glutamic acid	61.9 ± 9.9

[1] SE of mean (3 birds).

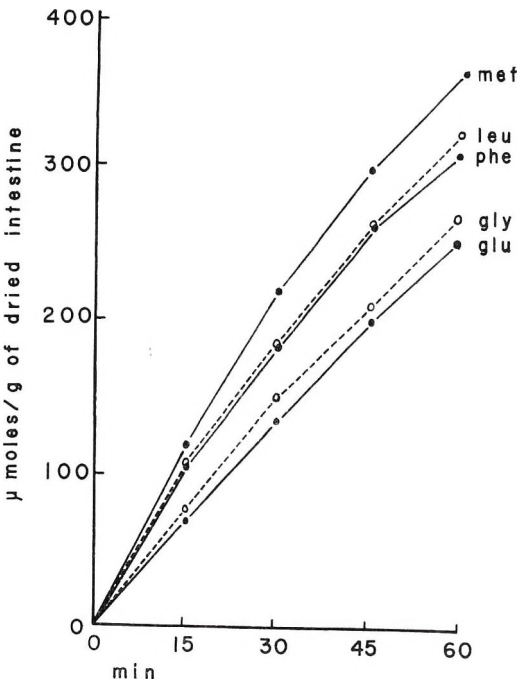

Fig. 1 Absorption of amino acids when a single L-amino acid was administered (10 μmoles/ml of circulating fluid).

of the absorption rate of methionine were not so great, showing 362.5 μmoles when administered singly, and 326.6 or 340.2 μmoles when administered with leucine or phenylalanine, respectively. These results indicate that methionine inhibits significantly the absorption of leucine and phenylalanine, but the inhibitory effect of leucine and phenylalanine on methionine absorption is not so great. However, when methionine and glutamic acid were given simultaneously, the absorption rate of glutamic acid was greatly inhibited (171.4 and 253.2 μmoles when administered with and without methionine, respectively), whereas the absorption rate of methionine appeared to be accelerated slightly (399.8 and 362.5 μmoles, with and without glutamic acid, respectively).

Table 2 indicates that when leucine, phenylalanine or methionine were administered singly or in combination, water absorption rates were almost constant, showing 13 ml during 60 minutes, but glutamic acid apparently accelerated the water absorption rate.

TABLE 2

Absorption rates of water and L-*amino acids when amino acids were administered singly or in combination (10 μmoles/ml of circulating fluid, each)*

Amino acid tested	No. of birds	Absorption rate/g of dried intestine				
		Water	Methionine	Leucine	Phenylalanine	Glutamic acid
		ml	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Leucine	5	12.7±1.6 [1]		322.5± 8.4		
Phenylalanine	5	13.2±1.2			310.3±12.8	
Glutanic acid	7	16.7±1.2				253.2± 6.2
Methionine	6	13.2±1.7	362.5±25.5			
Leucine+methionine	5	13.9±1.7	326.6±41.3	237.9±16.2 (26%) [2]		
Phenylalanine+methionine	5	12.9±1.7	340.2±16.2		231.5±26.2 (25%)	
Glutamic acid+methionine	5	17.7±1.6	399.8±44.0			171.4±18.9 (32%)

[1] Mean ± SE of mean.
[2] Values in parentheses indicate % of reduction in absorption rate of amino acid.

DISCUSSION

Experiment 1 (table 1) shows that considerable selectivity occurs in the amino acid absorption from the small intestine of birds when an equimolar mixture of 18 L-amino acids is introduced.

Orten (15) studied the amino acid absorption from an equimolar mixture of 18 L-amino acids in isolated segments (Thiry loop) of intestine of human subjects, and observed a marked selectivity in the absorption rate of the individual amino acids, showing rapid absorption of isoleucine, methionine, arginine, leucine and valine, and poor absorption of threonine, histidine, glycine and glutamic acid. Delhumeau et al. (16) studied the absorption pattern of individual amino acids from equimolar mixtures of 18 L-amino acids in tied-off segments of rat intestine in situ, and observed that cysteine, methionine, isoleucine, leucine and arginine belonged to a group of rapidly absorbed amino acids, whereas glutamic acid, aspartic acid, glycine and threonine constituted a group of slowly absorbed amino acids. Comparing the results of Orten, Delhumeau et al. and of this laboratory, rats, human beings and birds show the same absorption pattern for most of the amino acids: methionine, isoleucine and valine are rapidly absorbed, and glycine and glutamic acid are slowly absorbed. In birds, however, the absorption rate of methionine, the most rapidly absorbed amino acid, is only 1.5 times as great as that of the one absorbed most slowly, although the differences in absorption rates between the most rapid and the slowest in human beings or in rats are large, being 2.2 and 3.6 times, respectively.

Agar et al. (17) reported that L-amino acids, but not D-amino acids, with larger nonpolar side chains, such as trpytophan, methionine, leucine and isoleucine, were the most effective inhibitors of L-histidine uptake by isolated segments of rat intestine, whereas those with a charged side chain, such as glutamic acid, aspartic acid, arginine and lysine, were the weak inhibitors. From this result, it is assumed that amino acids which strongly inhibit the uptake of L-histidine belong to a group of rapidly absorbed amino acids in the present experiment, and in contrast, amino

acids which weakly inhibit the uptake of L-histidine belong to a group of slowly absorbed amino acids. According to Orten (15) and Delhumeau et al. (16), however, arginine was rapidly absorbed in human beings and rats, although in the present experiment arginine was slowly absorbed in birds. It is not clear why the absorption pattern of arginine shows such a marked difference in mammals and birds.

It was concluded that the absorption rate of amino acids was not dependent upon their molecular weight; when introduced separately, each amino acid has its own absorption rate which is a characteristic of that amino acid (fig. 1). This fact implies that some of the amino acids are actively absorbed, because if amino acids are absorbed only by diffusion as shown by Kratzer (4), the absorption rates of amino acids in definite concentration will be inversely proportional to their molecular weight.

Using an in vitro technique, Wiseman (7, 11, 18) reported that L-glutamic acid was not absorbed against a concentration gradient, although the majority of L-amino acids were actively absorbed. In the present experiment, L-glutamic acid was shown to be the amino acid absorbed most slowly; however, whether this amino acid was absorbed against a concentration gradient could not be ascertained from the data obtained.

Wiseman (11) using the everted small intestine of golden hamsters, observed that the active transport of glycine, L-proline and L-histidine was completely inhibited by equimolar amounts of L-methionine. Agar et al. (17) showed that the uptake of L-histidine was markedly inhibited by L-methionine or L-leucine in the intestinal segment of rats. Paine et al. (19) also reported that in chickens the presence of L-histidine appeared not to affect the absorption of L-methionine significantly, but some interference with L-histidine absorption was caused by L-methionine even though the data showed only borderline significance.

As shown in table 2, methionine has a considerable inhibitory effect upon the absorption of glutamic acid, leucine and phenylalanine. This result agrees with the observations of Wiseman, Agar et al. and Paine et al. Among the amino acids used in the present study the absorption rate of glutamic acid was reduced the most (32% of reduction rate during an hour's absorption), and the reduction in the absorption rate of leucine and phenylalanine was almost the same (26 and 25% respectively). This fact may imply that methionine has a greater inhibitory effect on the absorption of the acidic amino acids, such as glutamic acid, than it does on that of the neutral amino acids, such as leucine or phenylalanine.

Christensen et al. (20) observed that glutamic acid intensified the uptake of neutral amino acids in Ehrlich mouse ascites carcinoma cells in vitro. Agar et al. (17) also observed that the uptake of L-histidine into the segment of rat intestine was somewhat accelerated by L-glutamic acid. Table 2 shows that glutamic acid has a tendency to increase the methionine absorption, whereas leucine or phenylalanine depresses the absorption of methionine, although there are no significant differences.

As shown in table 2, the perfused intestine absorbed water from the circulating fluid at a rate of approximately 13 ml/g of dried intestine/hour, when the fluid contained methionine and leucine or phenylalanine, singly or in combination. However, when glutamic acid was present in the circulating fluid, water absorption was somewhat accelerated, showing 17 ml/g of dried intestine/hour. The increase of methionine concentration of the circulating fluid, and the final concentrations of methionine observed after an hour's absorption were almost the same when methionine was introduced with or without glutamic acid; showing 5.41 and 5.61 μmoles/ml of fluid, respectively. This fact suggests that diffusion plays a role in methionine absorption when methionine was administered with glutamic acid simultaneously, and consequently the increase of methionine absorption would occur.

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Effect of Dietary Protein Level Prior to Acute Starvation on Serum Proteins in the Rat [1,2]

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ABSTRACT The effect of acute starvation and refeeding on serum proteins was studied in rats receiving high and low protein diets. The levels for total proteins, albumin and α_1-globulin were significantly lower in the group receiving the low protein diet before starvation. No significant differences were observed in α_2-, β- and γ-globulin fractions. During starvation, the rats that had consumed the low protein diet showed a significant increase in total protein and albumin, a decrease in α_1- and β-globulin, and no significant change in α_2- and β-globulin. The rats fed the high protein diet, on the other hand, showed a progressive and significant decrease in total proteins, α_1- and β-globulin, an increase in γ-globulin and no significant change in albumin and α_2-globulin fractions. During refeeding, the rats fed the low protein diet prior to starvation, showed a significant decrease in albumin, and an increase in α_1-globulin when refed either diet. The rats fed the high protein diet prior to starvation, showed during refeeding, an increase in total proteins and albumin when refed the high protein diet and an increase in α_1-globulin when refed either diet. The plane of protein nutrition of an animal, therefore, influences the response to acute starvation.

It has been shown that the biochemical response of an animal to acute starvation is closely related to its nutritional status. This was demonstrated in relation to serum lipids when rats were fed, prior to starvation, diets containing 5 or 20% casein. In this case, the rats that had consumed the low protein diet showed, during starvation, an increase in the concentration of all serum lipid fractions, whereas rats fed the high protein diet had a marked and significant decrease (1).

Although the effect of starvation on serum proteins has been studied in animals and humans (2), there is no specific information in the literature about the effect of starvation on serum proteins in animals on different planes of nutrition. It is possible, as in the case of serum lipids, that the protein nutritional status of an animal might influence the response of its serum proteins to acute starvation. Furthermore, changes in serum protein fractions, especially those related to lipid transport, may contribute to an explanation of the changes in serum lipids during starvation.

The following study was undertaken to investigate the effect of acute starvation on serum proteins in growing rats that were fed a 20% casein diet as compared with those receiving a diet with only 5% casein. Serum lipid changes were also studied as an indicator of the starvation effect.

MATERIAL AND METHODS

Experimental design. Seventy-two weanling male rats of the Sprague-Dawley strain were divided into 2 groups, each having the same average weight of 76 g, and placed in individual cages with raised screen bottoms. One group was fed ad libitum a diet containing 5% casein and the other group, 20% casein during 8 weeks. The composition of these diets is shown in table 1. Body weight was recorded weekly and food consumption was determined during 3 days at the end of the sixth week. The rats fed the 20% casein diet consumed an average of 15.2 g/day, whereas the rats fed the 5% casein diet consumed only 8.3 g/day.

At the end of the eighth week, each dietary group was divided into 6 subgroups of the same average body weight. These

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[2] INCAP Publication I-361.

TABLE 1
Composition of diets

	5% Casein	20% Casein
Casein, g	5	20
Salt mixture,[1] g	4	4
Cellulose,[2] g	2	2
Cottonseed oil, ml	10	10
Cod liver oil, ml	1	1
Cornstarch, g	78	63
Vitamin mixture [3]		

[1] Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. G. Hart. J. Biol. Chem., *138*: 459, 1941; obtained from Nutritional Biochemicals Corporation, Cleveland.
[2] Alphacel, Nutritional Biochemicals Corporation.
[3] Each 100 g of diet were supplemented with 5 ml of a vitamin solution containing thiamine·HCl, 0.6 g; riboflavin, 0.6 g; nicotinic acid, 1 g; Ca pantothenate, 2 g; pyridoxine, 0.6 g; biotin, 2 mg; folic acid, 4 mg; vitamin B_{12}, 0.6 mg; inositol, 8 g; choline·HCl, 30 g; *p*-aminobenzoic acid, 6 g; menadione, 0.2 g; ethyl alcohol, 842 ml; and distilled water, to make 1 liter.

groups were treated in the following way: subgroups 1, 2, 3 and 4 within each dietary group were starved for periods of zero, 2, 4 and 6 days. Subgroups 5 and 6 within each dietary group were starved during 6 days and then refed during 8 days. In each dietary group, subgroups 5 were refed the 5% casein diet, and subgroups 6 were refed the 20% casein diet. Water was available at all times. The rats were decapitated at the end of each experimental period and blood for serum separation was collected.

Methods. Serum total lipids, cholesterol and lipid phosphorus were determined by micromethods adapted from those of Bragdon (3), Abell et al. (4), and Chen et al. (5), respectively. Serum total proteins were determined on the day of the sample collection, using the gradient tube method of Lowry and Hunter (6). Serum protein fractions were separated by paper electrophoresis by the method of Grassmann et al. (7) using 10 μliters of sample on Whatman no. 1 paper strips, and an acetate-barbital buffer, pH 8.6, with an ionic strength of 0.075. A current of 100 v was applied for 16 hours. The individual fractions were then determined colorimetrically after staining with Amido Black and eluting with 2 N sodium hydroxide solution. The method of Morrison and Slocum (8) was used for calculating the relative amounts of serum protein fractions. The absolute concentrations were obtained from the serum total proteins.

Analysis of variance (9) was applied in the statistical study of the results. The multiple range test of Duncan (10) was used in the comparison of individual means.

RESULTS

In table 2, the results in serum lipid fractions are shown. No significant differences in pre-starvation values were observed. Serum total lipids, cholesterol and lipid phos-

TABLE 2
Effect of dietary protein level prior to acute starvation and following refeeding on serum lipid concentration in the rat

Subgroup	No. days starved	Casein refed post-starvation	Body wt	Total lipids	Cholesterol	Lipid phosphorus
		%	*g*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
				5% Casein group		
1	0	—	115 ± 25 [1]	400 ± 28	85 ± 8	5.7 ± 0.5
2	2	—	—	483 ± 60*	127 ± 25**	9.4 ± 2.0**
3	4	—	—	421 ± 53	97 ± 18	6.9 ± 1.7
4	6	—	78 ± 14	369 ± 104	96 ± 18	5.8 ± 1.0
5	6	5	93 ± 18	444 ± 69	111 ± 17	7.1 ± 1.5
6	6	20	97 ± 18	476 ± 74*	119 ± 12*	8.3 ± 1.2*
				20% Casein group		
1	0	—	332 ± 42	399 ± 80	79 ± 15	6.0 ± 1.5
2	2	—	—	335 ± 97	65 ± 17	6.1 ± 0.7
3	4	—	—	280 ± 33**	46 ± 17**	3.7 ± 0.9**
4	6	—	272 ± 30	247 ± 34**	55 ± 16**	3.5 ± 0.9**
5	6	5	304 ± 42	405 ± 81**	95 ± 14**	7.6 ± 1.8**
6	6	20	329 ± 42	514 ± 86**	96 ± 8**	7.1 ± 2.1**

* Significant difference at $P \leq 0.05$, and ** significant difference at $P \leq 0.01$, when serum lipid starvation values are compared with initial subgroups no. 1 and refeeding values are compared with final starvation subgroups no. 4 within each dietary group.
[1] Mean ± sp.

TABLE 3

Effect of dietary protein level prior to acute starvation and refeeding on serum protein concentration in the rat

Sub-group	No. days starved	Casein refed post-starvation	Total proteins	Albumin	Globulin			
					α_1-	α_2-	β-	γ-
		%	*g/100 ml*	*g/100 ml*	*g/100 ml*	*g/100 ml*	*g/100 ml*	*g/100 ml*
				5% Casein group				
1	0	—	5.86 ± 0.14 [1]	2.45 ± 0.26	1.11 ± 0.16	0.68 ± 0.10	0.93 ± 0.12	0.67 ± 0.17
2	2	—	6.28 ± 0.66*	3.13 ± 0.35**	0.99 ± 0.16	0.69 ± 0.13	0.96 ± 0.16	0.50 ± 0.13
3	4	—	6.68 ± 0.28**	3.59 ± 0.27**	0.90 ± 0.21*	0.64 ± 0.11	1.01 ± 0.20	0.52 ± 0.11
4	6	—	6.28 ± 0.50*	3.33 ± 0.50**	0.87 ± 0.10*	0.67 ± 0.10	0.94 ± 0.09	0.47 ± 0.10*
5	6	5	5.78 ± 0.26	2.32 ± 0.08**	1.10 ± 0.19*	0.78 ± 0.18	0.85 ± 0.10	0.65 ± 0.26
6	6	20	5.99 ± 0.29	2.86 ± 0.19*	1.11 ± 0.14*	0.66 ± 0.03	0.87 ± 0.07	0.48 ± 0.04
				20% Casein group				
1	0	—	7.22 ± 0.32	3.28 ± 0.24	1.84 ± 0.36	0.60 ± 0.16	0.99 ± 0.14	0.51 ± 0.08
2	2	—	7.01 ± 0.27	3.20 ± 0.24	1.75 ± 0.13	0.56 ± 0.07	0.91 ± 0.24	0.59 ± 0.13
3	4	—	6.56 ± 0.24**	3.33 ± 0.19	1.33 ± 0.30**	0.57 ± 0.06	0.69 ± 0.10**	0.63 ± 0.22
4	6	—	6.37 ± 0.28**	3.19 ± 0.16	1.17 ± 0.11**	0.57 ± 0.06	0.72 ± 0.07**	0.71 ± 0.13*
5	6	5	6.53 ± 0.53	2.86 ± 0.19	1.41 ± 0.12*	0.65 ± 0.11	0.86 ± 0.20	0.74 ± 0.14
6	6	20	7.27 ± 0.42**	3.64 ± 0.32*	1.49 ± 0.27**	0.72 ± 0.06	0.86 ± 0.13	0.55 ± 0.19

* Significant difference at $P \leq 0.05$, and ** significant difference at $P \leq 0.01$, when starvation values are compared with initial subgroups no. 1, and refeeding values are compared with final starvation subgroups no. 4 within each dietary group.

[1] Mean ± SD.

phorus concentrations during starvation in the low protein group increased significantly in the first periods, and at the end they decreased toward the initial values. The high protein group, in contrast, showed a marked decrease in all lipid fractions. The final levels at the end of starvation were significantly higher in the group fed the low protein diet prior to starvation. When the group receiving the low protein diet was refed with either of the diets, an increase in all values was obtained. This increase, however, was significant only when the diet containing 20% casein was fed. When the animals in the high protein group were refed, after starvation, with either of the diets, a significant increase was also observed. In this group the animals refed with the high protein diet showed significantly higher values in total lipids than those refed the low protein diet. The cholesterol levels at the end of the refeeding period were higher in the group fed the 5% casein diet prior to starvation.

Serum total proteins and the electrophoretic fractions expressed in absolute concentrations are given in table 3. The pre-starvation levels for total serum proteins, albumin and α_1-globulin were significantly lower in the group receiving the 5% casein diet. No significant differences were observed in α_2-, β-, and γ-globulin fractions, although the γ-globulin concentration tended to be higher in this group.

The animals fed the low protein diet prior to starvation showed, during starvation, a significant increase in total proteins and albumin, and a decrease in α_1- and γ-globulin. No significant change was observed in α_2- and β-globulin. The high protein group, however, showed a progressive and significant decrease in total proteins, α_1- and β-globulin and an increase in γ-globulin. No significant change was observed in albumin and α_2-globulin fractions. The final values at the end of the starvation period were significantly lower for α_1- and γ-globulin and greater for β-globulin in the group fed the 5% casein diet prior to starvation.

During refeeding, the animals fed the 5% casein diet, prior to starvation, showed a significant decrease in albumin and an increase in α_1-globulin when refed either diet. The animals fed the 20% casein diet, prior to starvation, showed during refeeding an increase in total proteins and albumin when given the diet containing 20% casein and an increase in α_1-globulin when refed either diet.

DISCUSSION

The data on acute starvation presented here, demonstrate that the plane of protein nutrition in the rat affects significantly the response to acute starvation in terms of serum protein concentration. In the two dietary groups studied, the different behavior of the serum protein fractions indicates that hemoconcentration does not explain the changes which occurred. If changes in hemoconcentration had been the only operative mechanism, then all serum fractions would have changed proportionally in the same direction.

Rutman et al. (11), on the basis of the incorporation of methionine-^{35}S by liver slices, have demonstrated that the effect of fasting on the rat previously fed a low protein or protein-free diet is to increase the rate of incorporation. Fasting of rats previously fed a diet of higher protein content, however, reduces the rate of incorporation. If incorporation is assumed to result from net protein synthesis, then fasting produces dissimilar effects in protein synthesis in animals fed low or high protein diets.

Kaufmann and Wertheimer (12) have described a "fasting effect" on total nitrogen release from liver slices of rats fed diets of different protein content. They observed a proportionally greater nitrogen release in rats fed a protein-free diet or a restricted intake prior to starvation than rats fed a stock or a high protein diet. If the discharge of nitrogen from liver slices can be assumed to represent a release of protein in vivo, then there is a marked difference in the net amount of protein liberated into the circulation.

From the above discussion, it could be inferred that the increase in serum proteins observed during starvation in the group of rats fed the low protein diet prior to this stress, is due to a selective increase in protein synthesis, and protein mobilization. The results following refeeding could also be attributed to the same mechanism of protein synthesis and mobilization as

it has been observed that the total nitrogen release from liver slices of rats fed a low protein diet is lower than that of rats fed a normal one (12), and that the synthesis of proteins, as indicated by the incorporation of labeled methionine, is also greater in rats fed a normal or high protein diet (11).

The results on the serum lipid fraction presented in this paper confirm previous observations (1), which showed that the plane of nutrition of the rat affects the response to acute starvation. The fact that the 20% casein group of animals that had showed, during starvation, a marked decrease in all serum lipid fractions, also showed a decrease in α_1- and β-globulin concentration, is understandable in view of the association between both constituents in the blood plasma. Alpha- and β-globulin fractions are the main cholesterol carriers (13). Direct determinations of α- and β-lipoprotein concentrations during acute starvation may help to confirm this interpretation.

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Nutritive Value of Protein in High and Low Protein Content Sorghum Grain as Measured by Rat Performance and Amino Acid Assays [1]

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ABSTRACT The nutritive value of protein of 2 sorghum grain composites containing 7.9% and 11.8% protein, respectively, was compared on the basis of growth of rats and amino acid analyses. The high protein sorghum grain had higher percentages of all the 17 amino acids studied than did the low protein sorghum grain. In both grains, lysine was the most deficient amino acid, and content of sulfur-containing amino acids and threonine also was low. Calculated on an equal nitrogen basis, the dibasic amino acids, especially lysine, the sulfur-containing amino acids, and threonine levels of the high protein sorghum grain were lower than those of the low protein sorghum grain. Nutritive value of the protein of low protein sorghum grain was superior to that of a high protein sorghum grain, as shown both by growth of rats and amino acid assays. When lysine, histidine, and arginine were added to the high protein sorghum grain diet to adjust percentages of those amino acids in the protein similar to the content in the low protein sorghum grain diet, an increase in growth resulted.

Sorghum grains are used widely in feeds for poultry, swine and other farm animals. Factors such as environment and hybrids are known to cause variations in protein content of sorghum grains (1). With the expanding use of irrigation, fertilization, and hybrid seed in farming practices, the protein content of sorghum grain may vary more than formerly. There is a paucity of information on the effects of variation in protein content on the nutritive value of protein from sorghum grain. However, the nutritive value of corn has been studied more extensively. Sauberlich et al. (2) determined protein and 18 amino acids in a series of corn samples. Both variety and nitrogen fertilization influenced protein and amino acid content, but in the higher protein content corn, leucine, alanine, phenylalanine and proline made up a greater percentage of the protein than the others, whereas, arginine, glycine, lysine and tryptophan became a lesser percentage of the protein. Sauberlich et al.[2] stated that the main effect of nitrogen fertilization of corn was on quantity of protein rather than quality.

Using rats and chicks as test animals, Sauberlich et al. (3) determined the nutritive value of corns ranging from 6.8 to 13.6% protein. High protein corn was superior to low protein corn when fed as equal proportions of grain in the diet. For growth of their animals, both high and low protein corn were deficient in tryptophan and lysine, and the low protein corn was deficient also in isoleucine, threonine, and valine. When corn was fed on the basis of equal protein content in the diet, high protein corn was somewhat inferior to low protein corn.

Vavich et al. (4) observed that, as measured by chick growth, sorghum grain containing 10.5% protein was superior to sorghum grain containing 15.3% protein when fed at a constant level of protein in the diet. Using microbiological amino acid assays they found that the diet made with the 10.5% protein sorghum grain was low in lysine and arginine, and that made with the 15.3% protein sample was high in leucine and isoleucine.

The object of the present experiment was to determine the nutritive value of protein of high and low protein hybrid

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[2] Sauberlich, H. E., W-Y. Chang and W. D. Salmon 1951 Effect of nitrogen fertilization upon the biological value and amino acid content of corn. Federation Proc., *10:* 243 (abstract).

sorghum grain, as measured by growth of rats, and to correlate these data with the amino acid content of the protein.

EXPERIMENTAL

Two sorghum grains, both composites of several samples of the same relative protein content, were obtained from the Agronomy Department at Kansas State University. The low protein sorghum grain was 7.9% protein (N × 6.25) and the high protein sorghum grain was 11.8% protein by standard methods (5). Moisture content was 8.4% and 8.2%, respectively.

Seventeen amino acids were determined in each sample by ion exchange chromatography using a Beckman Amino Acid Analyzer, Model 120B. Samples were hydrolyzed in 6 N HCl for 22 hours at 110° under vacuum. The sample was filtered and evaporated to dryness; water was added and evaporation repeated twice. Sodium citrate buffer, pH 2.2, was added and the samples were stored in a frozen condition until analyzed. Results of these analyses were used in balancing amino acid and protein content of diets, as indicated later.

Thirty 28-day-old albino rats distributed in a randomized complete block design were used in a 4-week growth study. Blocks consisted of rats of the same sex within a 5-g weight range. Feed and water were supplied ad libitum.

Six different diets, shown in table 1, were fed to groups of 5 rats caged individually. Rats were housed in metal screen-bottom cages in a room maintained at a temperature of 26 to 28°. Sorghum grain plus the indicated amino acids were the only sources of protein in diets 1, 2, 3 and 4. Glutamic acid and additional glycine were added to diets 1, 2 and 4 so that those rations would contain approximately the same quantity of N as diet 3. Na_3PO_4 was added to diets containing starch to adjust all diets to a similar phosphorus content. Starch was substituted for part of the high protein sorghum grain in diets 2 and 3 so that those diets had the same total protein content as diet 1, made from low protein sorghum grain. Diet 3 contained additional lysine, histidine, and arginine to raise the content to the same levels as in diet 1. Diets 5 and 6 were supplemented with casein to 14% protein. Starch was substituted for some sorghum grain in diet 6 so that diets 5 and 6 contained the same amount of grain protein.

Analysis of variance and the LSD means separation method were made by standard methods (7). The level of significance used was, $\alpha = 0.05$.

TABLE 1

Diet composition

Diet	1	2	3	4	5	6
Protein level of sorghum grain	Low	High	High	High	Low	High
Protein content,[1] %	7.6	7.6	7.6	11.5	14.0	14.0
	%	%	%	%	%	%
Sorghum grain	96.0	64.2	64.2	96.0	87.5	58.5
Starch	—	31.65	31.65	—	—	28.85
Casein	—	—	—	—	8.7	8.7
Na_3PO_4	—	0.15	0.15	—	—	0.15
Salt	0.3	0.3	0.3	0.3	0.3	0.3
Dicalcium phosphate	2.0	2.0	2.0	2.0	2.0	2.0
Vegetable oil	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix [2]	0.025	0.025	0.025	0.025	0.025	0.025
Vitamin premix [3]	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	0.073	0.073	0.039	0.073	—	—
Glutamic acid	0.073	0.073	—	0.073	—	—
Lysine	—	—	0.048	—	—	—
Histidine	—	—	0.010	—	—	—
Arginine	—	—	0.030	—	—	—

[1] Diets 1, 2, 3, 4 had additional purified amino acids added as indicated.
[2] CCC All Purpose Trace Mineral Premix, 5% Zinc, Calcium Carbonate Company, Quincy, Illinois.
[3] Vitamin premix prepared using pure vitamins and vitamin concentrates on a grain carrier; 0.5% of premix in diet supplied recommended levels in the diet (6).

RESULTS AND DISCUSSION

Data from the amino acid determinations are shown in table 2. The dibasic amino acids, especially lysine, the sulfur-containing amino acids and threonine appeared to be the amino acids most deficient in both the low and high protein sorghum grains. All the amino acids studied were present in a higher percentage in the high protein sorghum grain. Lysine increased the least from the low to high protein grain.

All the essential amino acids, with the exception of leucine and phenylalanine, made up a smaller percentage of the protein of high protein sorghum grain than of the protein of low protein sorghum grain (table 2). These values indicate that protein of the low protein sorghum grain would be expected to have the higher nutritive value.

Body weight gains of the rats for the 4-week period are shown in tables 3 and 4. There was a significant effect of diets on growth (tables 3 and 4). Casein-supplemented diets produced much greater growth than did unsupplemented diets. When low and high protein sorghum grains were fed as the same percentage of the diet (diets 1 and 4) there was no significant difference in growth, although the rats fed high protein sorghum grain grew slightly more. Fed on an equal protein basis, the low protein sorghum grain caused significantly greater growth than did high protein sorghum grain (diets 1 and 2). When lysine, histidine and arginine were added to diet 2, to produce diet 3 with a distribution of those amino acids similar to diet 1, growth increased, but not significantly.

These results might be explained partially by data in table 5 showing percentages of the required levels of essential amino acids that were supplied by the diets. Almquist (8) states that "one serious amino acid deficiency is enough to cause a failure of the entire diet." When related to this assumption the results can be explained. Lysine is the most limiting amino acid in the diets without casein supplementation. Thus the high protein sorghum grain (diet 4) did not produce significantly more growth than did the low protein sorghum grain (diet 1) when fed as equal amounts of grain in the diets. In casein-supplemented diets (5 and 6) the sulfur-containing amino acids are limiting; but based on composition, these diets should be superior to the others.

In somewhat related studies Dobbins et al. (9) fed fattening pigs and rats equal

TABLE 2

Amino acid composition of the low and high protein sorghum grains

	Low protein		High protein	
	% of sample [1]	*% of protein* [2]	*% of sample*	*% of protein*
Lysine	0.19	2.48	0.21	1.81
Histidine	0.18	2.28	0.25	2.10
Arginine	0.27	3.54	0.35	3.01
Aspartic acid	0.51	6.66	0.75	6.43
Threonine	0.25	3.27	0.35	2.96
Serine	0.34	4.39	0.49	4.15
Glutamic acid	1.63	21.09	2.57	21.96
Proline	0.64	8.33	0.96	8.19
Glycine	0.25	3.25	0.32	2.70
Alanine	0.71	9.14	1.15	9.85
Half-cystine	0.11	1.44	0.18	1.56
Valine	0.39	5.01	0.57	4.86
Methionine	0.09	1.22	0.14	1.17
Isoleucine	0.31	4.01	0.46	3.95
Leucine	1.04	13.52	1.70	14.53
Tyrosine	0.20	2.56	0.33	2.83
Phenylalanine	0.39	5.04	0.60	5.12

[1] Percentage of the individual amino acids in the sample; 0.21% and 0.33% ammonia in low and high protein grain samples, respectively.

[2] The percentage of protein was calculated by dividing the content of the individual amino acid by the total of the 17 amino acids + ammonia recovery from the analysis.

TABLE 3

Data and analysis of variance of weight gains of rats fed for a 4-week period

Block	Diet				Total	Average
	1	2	3	4		
	g	*g*	*g*	*g*	*g*	*g*
1 (F, 79–83 g) [1]	19	1	3	27	50	12.5
2 (F, 70–73 g)	17	3	11	27	58	14.5
3 (M, 78–83 g)	22	5	23	8	58	14.5
4 (M, 77–80 g)	13	10	4	19	46	11.5
5 (M, 90–94 g)	17	3	14	16	50	12.5
Total	88	22	55	97		
Average	17.6	4.4	11.0	19.4		

Analysis of variance

Source of variation	df	Sum of squares	Mean square
Diets	3	600.2	233.4*
Block	4	28.8	7.2 n.s.
Error	12	584.8	48.7
Total	19	1313.8	

LSD = 9.5

[1] Sex and range of beginning weights.
* Level of significance = 5%.

TABLE 4

Analysis of variance of weight gains of rats fed for a 4-week period

Block	Diet		Total	Average
	5	6		
	g	*g*	*g*	*g*
1 (F, 79–83 g) [1]	102	85	187	93.5
2 (F, 70–73 g)	80	74	154	77.0
3 (M, 78–83 g)	149	112	261	130.5
4 (M, 77–80 g)	133	115	248	124.0
5 (M, 90–94 g)	125	111	236	118.0
Total	589	497		
Average	117.8	99.4		

Analysis of variance

Source of variation	df	Sum of squares	Mean square
Diets	1	846.4	846.4*
Block	4	4063.4	1015.8*
Error	4	260.6	65.2
Total	9	5170.4	

[1] Sex and range of beginning weights.
* Level of significance = 5%.

quantities of corn containing 7.7, 9.1, and 12.8% crude protein plus supplement to raise all rations to 15% protein. They found that the lower protein corn gave best results. Sauberlich et al. (3) reported that growth of rats and chicks fed equal quantities of protein in the diet was better with low protein than with high protein corn. Dobbins et al. (10) and Mitchell et al. (11) reported that the biological value decreased as protein content of corn increased.

TABLE 5

Percentage of the requirements of essential amino acids for the growing rat supplied by the various diets [1,2]

Diet	1	2	3	4	5	6
Protein level of sorghum	Low	High	High	High	Low	High
Protein, %	7.6	7.6	7.6	11.8	10.0	10.0
	%	%	%	%	%	%
L-Histidine	57	53	57	80	100 [3]	100
L-Lysine	20	14	20	22	86	81
L-Leucine	100	100	100	100	100	100
L-Isoleucine	60	60	60	88	100	100
L-Phenylalanine [4]	68	70	70	100	100	100
L-Methionine [5]	30	30	30	43	72	72
L-Threonine	48	44	48	68	100	100
L-Valine	53	51	51	79	100	100
L-Arginine	100	100	100	100	100	100

[1] Minimum requirement, National Research Council ((6) table 2, p. 54).
[2] Tryptophan was not determined in this experiment.
[3] Some of the values were more than 100% of requirement.
[4] L-Tyrosine equivalent made up one-third of the L-phenylalanine.
[5] L-Cystine equivalent made up one-half of the L-methionine.

There was not a significant block effect in diets 1, 2, 3 and 4, but a significant block effect was found in the casein-supplemented diets. The probable reason for this is that rats fed the casein-supplemented diets grew much more, allowing differences in growth rates of female and male rats to become apparent. Growth with the other diets was not sufficient to cause this effect.

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Relation of Vitamin B_{12} and One-carbon Metabolism to Hydrocephalus in the Rat [1,2,3]

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ABSTRACT The purpose of this investigation was to examine the relationship of a number of factors involved in 1-carbon metabolism to the induction of hydrocephalus in the rat. Animals raised with a diet deficient in vitamin B_{12} gave birth to young with congenital hydrocephalus. The addition of an intestinal antibiotic, neomycin, to the dams' diets or the parenteral administration of vitamin B_{12} antagonists did not markedly increase the incidence of congenital abnormalities. The incidence of congenital hydrocephalus was markedly increased by the inclusion in the diet of X-methyl folic acid, a folic acid antagonist. A higher incidence was obtained, however, when females were fed a diet deficient in vitamin B_{12} and choline. Methionine and sarcosine in equivalent methyl groups did not offer the same protective effect as did choline. Abnormalities were similar in newborn animals from vitamin B_{12}-deficient dams raised with the folic acid antagonist diets or with the choline-deficient diets. Lesions noted in offspring from these animals included: 1) stunted embryonic growth, 2) hydrocephalus, 3) hydro-ureter and hydronephrosis, 4) umbilical hernia, 5) spina bifida and cranioschisis, 6) harelip, and 7) skeletal defects.

While studying the nutritional requirements for reproduction and lactation in the rat, Richardson and Hogan (1) observed spontaneous cases of hydrocephalus in newborn animals. Approximately 2% of the offspring born to dams raised with a purified ration were hydrocephalic. The original diet consisted of casein and all of the vitamins available at that time, but it was later shown by O'Dell et al. (2) that supplementation of the diet with folic acid would largely prevent the occurrence of the congenital hydrocephalus. These investigators reported that X-methyl folic acid, an antimetabolite, added to soybean oil meal diets during successive gestations resulted in an increased incidence of hydrocephalic offspring compared with animals fed a casein diet (3). Vitamin B_{12} supplementation was later shown to prevent completely the occurrence of congenital abnormalities in newborn rats whose dams had received the vegetable protein diet (4). The X-methyl folic acid was considered to inhibit vitamin synthesis by intestinal microorganisms.

Newberne and O'Dell (5) were able to produce congenital hydrocephalus in rats by a maternal vitamin B_{12} deficiency; the diets did not contain any antagonist and were supplemented with 5 mg of folic acid and 1 g of choline chloride/kg of diet. Whereas the incidence of hydrocephalus in the folic acid antagonist diets of Hogan et al. (3) was 20%, a 10% incidence was noted when the ration was deficient only in vitamin B_{12} (5). Spina bifida, anophthalmia, harelip, cleft palate and edema were noted with the antagonist diet, but none of these lesions was observed in the newborn animals raised with the vitamin B_{12}-deficient ration without the antagonist.

Because of the differences reported by Hogan et al. (3) and Newberne and O'Dell (5), an attempt has been made to study further, congenital abnormalities in vitamin B_{12}-deficient neonatal rats by determining which accessory dietary factors might influence the incidence or severity of lesions.

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GENERAL METHODS

Weanling albino rats of the Caesarean Derived (CD) Sprague-Dawley strain were obtained from the Charles River Breeding Farm. The animals were housed in air conditioned animal quarters; food and water were supplied ad libitum. Three female animals were housed together in large, raised, screen-wire cages and fed either a vitamin B_{12}-deficient diet or a vitamin B_{12}-supplemented diet during the growth period.[5] The 30% protein diet shown in table 1 was supplemented with 0.3% choline chloride and 0.1% DL-methionine during the growth period; vitamin B_{12} was added to the control diet at the rate of 50 μg/kg of diet. At 13 weeks of age, the rats were placed on the various experimental rations. Females were fed the experimental ration for 4 weeks prior to breeding. Male animals fed a commercial ration were housed separately during their growth period and then placed with females, one male per cage. Females were allowed to litter in their cages, and the offspring were collected as soon as possible after delivery. Hydrocephalus was determined by incising formalin-fixed specimens.

TABLE 1

Composition of basal diets

	1	2
	% of diet	
Sucrose	55	65
Soybean protein [1]	30	20
Salts [2]	5	5
Cottonseed oil [3]	10	10
Vitamins [4]	*mg/kg of diet*	
Thiamine·HCl	16	16
Pyridoxine·HCl	16	16
Riboflavin	16	16
Ca pantothenate	40	40
Nicotinamide	12	12
Folic acid	5	5
Inositol	250	250
dl-α-Tocopheryl acetate [5]	112	112
	IU/kg of diet	
Vitamin A	22,800	22,800
Vitamin D	2,280	2,280

[1] Alpha Protein, Central Soya Company, Chicago (1.05% methionine).
[2] Hegsted et al. J. Biol. Chem., *138*: 459, 1941.
[3] Wesson oil, Wesson Sales Company, Fullerton, California.
[4] Vitamin B_{12} (50 μg/kg of diet) was added to the control diets.
[5] Hoffmann-LaRoche, Inc., Nutley, New Jersey.

Three separate experimental trials were conducted. The first trial was performed to determine the effects of feeding an intestinal antibiotic or vitamin B_{12} antagonists (monobasic acids of vitamin B_{12}) (6, 7). In the second trial the effect of a choline deficiency and the effect of feeding the folic acid antagonist, X-methyl folic acid, were determined. The third trial examined effects of lowering dietary protein levels, of substituting various methyl donors for choline, of parenteral administration of vitamin B_{12} antagonists and of feeding X-methyl folic acid in a choline-deficient diet.

METHODS AND RESULTS

Experimental trial 1

During the growth period, changes in body weight were measured weekly. Averages for the groups fed the vitamin B_{12}-deficient growth diet (diet C, table 2) and the control diet (diet D) are presented in figure 1 as a growth rate curve. When a 30% soybean protein diet contained 0.1% DL-methionine and 0.3% choline chloride, the growth rate of vitamin B_{12}-deficient rats was found to be similar to that of control animals. After the animals reached maturity the vitamin B_{12}-deficient group was divided into 3 subgroups and either maintained with the growth diet (diet C), given a diet containing neomycin (diet B), or fed a ration containing vitamin B_{12} antagonists (diet A). The adult vitamin B_{12}-deficient animals gave birth to hydrocephalic young; the control animals did not. Inclusion of an intestinal antibiotic, neomycin, in the diet did not greatly alter the incidence of hydrocephalus, whereas supplementation with monobasic acids of vitamin B_{12} caused infertility of that group. One apparently normal litter from the vitamin B_{12} antagonist group was obtained after the males in the cages were exchanged with those maintained with a commercial ration. The incidence of hydrocephalus is shown in table 2.

[5] Vitamin B_{12} antagonists were generously supplied by Dr. E. Lester Smith, Glaxo Research Laboratories, Ltd., Greenford, Middlesex, England. The X-methyl folic acid was kindly donated by Dr. E. H. Dearborn of Lederle Laboratories, Pearl River, New York. The soybean protein, Alpha Protein, was supplied through the courtesy of Central Soya Company, Chicago.

TABLE 2

Incidence of hydrocephalus (trial 1)

Diet (Ingredients added to basal diet 1, see table 1)		No. females fed diet	No. litters born	Avg no. animals/ litter	Newborns found dead	Avg wt newborn animals	Litters with hydrocephalic animals	Hydrocephalic animals
	mg/kg diet				%	*g*	%	%
Diet A		6	1	8.0	—	—	0	0
DL-methionine	3000							
choline chloride	1000							
monobasic acids of vitamin B_{12}	10							
Diet B		6	15	6.8	6.7	5.2	13.3	2.0
DL-methionine	3000							
choline chloride	1000							
neomycin sulfate	125							
Diet C		60	149	6.6	5.1	5.3	9.8	1.3
DL-methionine	3000							
choline chloride	1000							
Diet D		30	86	6.7	5.7	5.7	0	0
DL-methionine	3000							
choline chloride	1000							
vitamin B_{12}	0.05							

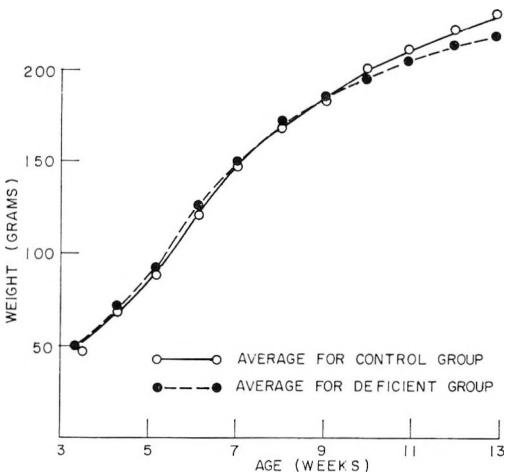

Fig. 1 Growth rate for vitamin B_{12}-deficient and control animals.

Experimental trial 2

The experimental procedure in trial 2 differed from that of trial 1. In trial 2 the mature rats were maintained with the growth diet (diet C, table 3) or with the control diet (diet D) for 2.5 months before being divided into subgroups. During this time, the females of trial 2 gave birth to one and sometimes two litters. All vitamin B_{12}-deficient females that had previously given birth to hydrocephalic young were maintained with a diet deficient in vitamin B_{12} (diet C, subgroup 1-a; table 3). One-third of the deficient animals were given a ration deficient in vitamin B_{12} but containing 10 mg of X-methyl folic acid/kg of diet (diet E, subgroup 1-b; table 3). The animals in the third subgroup were given a vitamin B_{12}-deficient ration which contained no choline (diet F, subgroup 1-c). The control animals were also divided into 3 subgroups (2-a, 2-b, 2-c) and fed diets D, G, or H.

The results (table 3) demonstrated that the addition of X-methyl folic acid to the diet caused a marked increase in the incidence of hydrocephalus (subgroup 1-b); however, a greater incidence of hydrocephalus occurred when the dams' diets were made deficient in choline as well as in vitamin B_{12} (subgroup 1-c). Females maintained with the folic acid antagonist-containing diet or the choline-deficient diets gave birth to offspring which were often small. Small embryos weighing less than 4 g were most common from dams fed the vitamin B_{12}-choline-deficient diets. Umbilical hernia and limb abnormalities were noted in hydrocephalic animals born to dams receiving the folic acid antagonist.

Samples of liver from adult and newborn animals were collected at the time

TABLE 3

Incidence of hydrocephalus (trial 2)

Group	Sub-group	Diet (Ingredients added to basal diet 1, see table 1)		No. litters	Total no. newborn	No. found dead	Avg wt newborn animals	No. animals weighing under 4 g	Litters hydro-cephalic	Newborn animals hydro-cephalic
			mg/kg diet				*g*		%	%
1		Diet C (before group subdivision)		17	161	9	5.6	0	5.8	0.6
		DL-methionine	3000							
		choline chloride	1000							
2		Diet D (before group subdivision)		7	54	1	6.1	0	0	0
		DL-methionine	3000							
		choline chloride	1000							
		vitamin B_{12}	0.05							
1	a	Diet C		16	115	5	5.4	0	18.0	6.0
		DL-methionine	3000							
		choline chloride	1000							
1	b	Diet E		19	101	12	6.1	3	33.3	13.8
		DL-methionine	3000							
		choline chloride	1000							
		X-methyl folic acid	10							
1	c	Diet F		15	72	16	5.8	12	60.0	37.5
		DL-methionine	3000							
2	a	Diet D		3	28	0	6.0	0	0	0
		DL-methionine	3000							
		choline chloride	1000							
		vitamin B_{12}	0.05							
2	b	Diet H		8	62	5	5.6	0	0	0
		DL-methionine	3000							
		choline chloride	1000							
		X-methyl folic acid	10							
		vitamin B_{12}	0.05							
2	c	Diet G		6	36	6	6.4	0	0	0
		DL-methionine	3000							
		vitamin B_{12}	0.05							

of necropsy. These were analyzed for vitamin B_{12} by the method outlined by the USP (8). Results (table 4) indicated the following: 1) animals raised with a vegetable protein diet deficient in vitamin B_{12} had low levels of vitamin B_{12} when compared with the control animals; 2) the addition of X-methyl folic acid to the diet of vitamin B_{12}-deficient animals did not cause a further reduction in liver levels of the vitamin; this suggested that the folic acid antagonist might exert a systemic action rather than inhibit the intestinal synthesis of vitamin B_{12}; 3) there were no differences in liver levels of vitamin B_{12} between hydrocephalic and nonhydrocephalic littermates; and 4) there were no differences noted in vitamin B_{12} levels between deficient newborn animals from hydrocephalic litters and nonhydrocephalic litters. It should be noted that animal no. 1 from the vitamin B_{12}-deficient adult group gave birth to only one hydrocephalic litter, whereas animal no. 2 gave birth to numerous hydrocephalic litters. The liver levels of vitamin B_{12} were essentially the same in all deficient animals.

Experimental trial 3

In contrast with the 30% protein diets used in trials 1 and 2, the basic ration of trial 3 contained only 20% protein. Three separate control groups were used. The first control group was fed the basic 20% protein diet supplemented with choline and vitamin B_{12} (diet 1, table 5). The second was fed a choline-deficient and vitamin B_{12}-supplemented diet (diet J). The third was fed a vitamin B_{12}-deficient and choline-supplemented diet (diet K). A fourth group was given diet K and, in addition, received intraperitoneally 50 μg of the vitamin B_{12} antagonists (monobasic acids of vitamin B_{12})/week (total of 0.9 mg/animal). Other groups were given rations supplemented with sarcosine hydrochloride, DL-methionine, and DL-homocysteine (diet L); DL-methionine and ethanolamine (diet M); or X-methyl folic acid (diet N). One mole of DL-methionine and 2 moles of sarcosine hydrochloride were considered to have methyl groups equivalent to 1 mole of choline. Three moles of DL-methionine were considered equivalent to 1 mole of choline in methyl groups.

Female animals receiving the vitamin B_{12} antagonists intraperitoneally gave birth to young which were normal in all respects. For this reason, this group of animals was included in table 5, with those receiving the vitamin B_{12}-deficient diet but not receiving the antagonist. No congenital abnormalities were detected grossly in the animals receiving the vitamin B_{12}-deficient ration supplemented with choline. A high incidence of congenital malformations was noted when either sarcosine and methionine or methionine alone was substituted for choline. The addition of X-methyl folic acid to the choline-deficient, vitamin B_{12}-deficient ration caused the adults to lose weight and to become infertile (see table 5).

When the protein content of the basal diet was reduced from 30 to 20%, the females receiving the choline-deficient but methionine - supplemented rations gave birth to offspring which had multiple con-

TABLE 4

Vitamin B_{12} analysis (trial 2)

Group	Litter	Liver vitamin B_{12}
		mμg/g of liver
Adult animals		
Control (D)		143–202
Vitamin B_{12}-deficient (subgroup 1-a)		6–7
Vitamin B_{12}-deficient with X-methyl folic acid added (subgroup 1-b)		9–10
Newborn animals		
Vitamin B_{12}-deficient newborn animals from dam in subgroup 1-a	hydrocephalic	1–2
	nonhydrocephalic	3–4

TABLE 5

Incidence of hydrocephalus (trial 1)

Diet (Ingredients added to basal diet 2, see table 1)		No. females fed diet	No. litters born	Litters with hydrocephalic young	No. newborn animals	Newborn animals hydrocephalic
	mg/kg diet			%		%
Diet I		8	8	0	42	0
choline bitartrate	1814					
vitamin B_{12}	0.05					
Diet J		8	7	0	64	0
vitamin B_{12}	0.05					
Diet K		16	7	0	64	0
choline bitartrate	1814					
Basal diet 2 (vitamin B_{12}- and choline-deficient)		8	3	33	16	25
Diet L		16	7	43	36	22
DL-methionine	1069					
sarcosine·HCl	1312					
DL-homocysteine	1452					
Diet M		16	8	38	65	8
DL-methionine	3207					
ethanolamine	440					
Diet N		16	0	—	—	—
X-methyl folic acid	10					

genital abnormalities. Hydrocephalus was by far the most common abnormality observed; however, on occasion the hydrocephalic animals had other defects as well. These included hydronephrosis, hydro-ureter, harelip, umbilical hernia, spina bifida, cranioschisis, and skeletal defects.

DISCUSSION

Weissbach et al. (9) demonstrated in partially purified fractions from animal liver that the methyl group of methyl B_{12} can be transferred to homocysteine. Buchanan et al. (10) studied the enzyme system in pig liver and provided convincing evidence that the pig liver enzyme is similar to the bacterial vitamin B_{12}-containing enzyme, which transfers a methyl group from 5-methyl-tetrahydrofolic acid to homocysteine to yield methionine and tetrahydrofolic acid. It was suggested by Herbert and Zalusky (11) that in vitamin B_{12}-deficient subjects, pteroylglutamic acid was converted to a metabolically useful form (probably N-5-methyl-tetrahydrofolic acid), which "piles up" in the serum because vitamin B_{12} is required for its normal utilization. The accumulation of 5-methyl-tetrahydrofolic acid occurs because the reaction catalyzed by the enzyme 5,10-methylene-tetrahydrofolate reductase probably operates irreversibly under physiological circumstances (10). Therefore, in vitamin B_{12} deficiency there appears to be a block in conversion of 5-methyltetrahydrofolic acid to methionine and a failure to regenerate tetrahydrofolate. The failure to regenerate tetrahydrofolate and its derivatives could cause alterations in purine and thymidine methyl synthesis as well as affect the catabolism of histidine.

It has always been assumed that the teratogenic effects of diets deficient in either folic acid or vitamin B_{12} were the consequence of the participation of these 2 vitamins in nucleic acid metabolism. The results of the experiments presented here show that deletion of choline from a vitamin B_{12}-deficient diet greatly increases the incidence of congenital abnormalities in neonatal rats. Choline in the form of phospholipids is important as a structural component of cells, in lipid transport be-

tween tissues, and probably in the metabolism of fatty acids in the liver. Methionine in amounts equivalent to choline in methyl groups would not offer the same protective effects. Thus, a choline-containing compound was probably a critical metabolic intermediate responsible for preventing the congenital abnormalities observed. Vitamin B_{12} could increase the availability of this intermediate so that the demands of pregnancy could be satisfied. It is still possible, however, that a deficiency of choline caused more methyl groups to be utilized for the synthesis of the missing metabolite and that this in turn decreased the methyl groups available for other functions such as purine and pyrimidine synthesis.

The sarcosine diets gave a high incidence of hydrocephalus; however, it was probably not different from that observed with methionine. The data were not inconsistent with the presence of a vitamin B_{12}-containing enzyme in the terminal chain of methionine synthesis from methyltetrahydrofolic acid; however, there were implications that the action of the vitamin may be more involved. This idea is supported by the synergistic action of X-methyl folic acid and vitamin B_{12}. If it is assumed that the X-methyl folic acid is acting as a systemic folic acid antagonist, then it is plausible to postulate that vitamin B_{12} and folic acid gave some independent action.

The feeding of an intestinal antibiotic, the injection of vitamin B_{12} antagonists, and the measurement of liver levels of vitamin B_{12} all indicated that factors other than a deficiency of vitamin B_{12} are concerned with production of congenital hydrocephalus in neonatal rats. The fact that the incidence and severity of lesions are increased when vitamin B_{12}-deficient diets are made deficient in choline or when X-methyl folic acid is added stresses the role of this vitamin in 1-carbon metabolism. Further research must be conducted to determine whether the primary metabolic aberration is in nucleic acid synthesis or is concerned with choline per se.

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Some Qualitative Amino Acid Needs of Adult Swine for Maintenance

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ABSTRACT A purified diet containing crystalline L-amino acids at a level to simulate the essential amino acid composition of a 3% crude protein, corn-soybean meal mixture was used to qualitatively evaluate some amino acid requirements for maintenance of the nongravid, postestrual gilt. No external symptoms of amino acid deficiency were noticed as a result of complete omission from the diet of those amino acids found to be required for maintenance. Diet consumption although controlled and not ad libitum was excellent regardless of the pattern of amino acids imposed. Neither protein-depleted nor protein-nondepleted gilts required arginine or histidine for maintenance. Hemoglobin was not significantly reduced by omission of either of these 2 amino acids from the diet. With a leucine-void diet, nitrogen balance exceeded 1 g/day, indicating that leucine, too, was nonessential for maintenance. In order of decreasing effect, a dietary void of threonine, isoleucine, lysine or phenylalanine reduced nitrogen balance. Nitrogen utilization was extremely poor with diets containing a void of either threonine or isoleucine. Regardless of whether dispensable or indispensable for maintenance, an amino acid omitted from the otherwise complete diet caused a lowered plasma level of this amino acid.

An amino acid may be essential for growth, reproduction or production but nonessential for maintenance. It has been adequately demonstrated in ^{15}N and ^{14}C labeling experiments that mammalian species can utilize nitrogen of inorganic origin and carbon derived from glucose, formate or acetate to form certain amino acids (1–3).[3] The question, then, becomes whether enough of a given amino acid can be synthesized to meet the requirement for maintenance.

Because neither qualitative nor quantitative information is available on the amino acid needs for maintenance of swine, the present study was undertaken. The objectives of this series of experiments were 1) to establish a suitable purified, crystalline amino acid diet that could serve as a positive control in future experiments, and 2) to ascertain which amino acids are required for maintenance of the adult nongravid gilt.

EXPERIMENTAL PROCEDURE

Nongravid crossbred gilts showing estrus were selected from the general herd on the basis of sire, age (10 to 11 months), weight (140 to 150 kg) and overall condition. They were allotted at random to metabolism cages similar in construction to those used by Mitchell and Kick (4). An experimental period of 14 days was used with total excreta collections being made on the last 5 to 7 days of each period. Unless otherwise indicated, the daily diet allotment per gilt was 1.82 kg which was divided equally into 2 feedings. Water was supplied twice daily in addition to that given with the diet (weight ratio of 1:1) so that water consumption was close to ad libitum.

Urine was filtered through glass wool into 10-liter plastic vessels containing 25 ml of concentrated HCl. Each daily urine collection was diluted to 6 liters and a 100-ml aliquot was taken. Accumulated aliquots were stored under toluene in a refrigerator until analysis was performed. A 5-day fecal collection was determined by detection of a ferric oxide marker (1% of the diet, fed at 5-day intervals) in the feces. Feces were collected daily, placed in

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[1] Present address: Eli Lilly and Company, Greenfield, Indiana.

[2] This paper represents part of a thesis submitted by the senior author to the Graduate College of the University of Illinois, in partial fulfillment of the requirements for the Ph.D. degree.

[3] Levy, L., and M. J. Coon 1952 Histidine synthesis in yeast and human liver. Federation Proc., 11: 248 (abstract).

plastic bags, and frozen. Upon termination of an experiment the feces were placed in a forced hot-air oven until dry, after which they were allowed to equilibrate with air moisture for 48 hours. Following drying and equilibration, the feces were weighed and ground in a Wiley mill equipped with a 40-mesh screen. Feed samples were ground in similar fashion. Representative feed, fecal and urine samples were analyzed for nitrogen as outlined by AOAC (5).

A completely purified diet (table 1) containing crystalline L-amino acids at a level to simulate the essential amino acid composition of a 3% crude protein corn-soybean meal reference diet (table 2) was used in most of the studies to be reported. L-Methionine and L-phenylalanine were added to supply the desired level of total sulfur and total aromatic amino acids, respectively. L-Glutamic acid was used to bring the total nitrogen content of the diet up to 0.48% (3% crude protein equivalent) and thereby provide nonspecific nitrogen. Rippel et al. (6) demonstrated that the gravid gilt could effectively use glutamic acid for this purpose. A preliminary assay revealed that a dietary level of 0.10% phenylalanine would maximize nitrogen retention; therefore, this amino acid was revised from an original level of 0.26% to 0.10%.

Since the reference diet (weight ratio of 4.1:1 of corn to soybean meal) was to be the basis of future work, it seemed desirable to obtain proof of the protein and energy adquacy of this diet for the nongravid gilt. Rippel et al. (7) demonstrated earlier that a diet similar in amino acid composition but slightly lower in energy would provide adequate protein for maintenance of the gravid gilt. To provide information on the endogenous nitrogen excretion of the nongravid gilt, a nitrogen-free diet (table 1) was also evaluated.

The energy values of Diggs et al. (8) provide the best estimate of the energy content of the diets in question. Since cornstarch, corn and soybean meal (50% CP) have very nearly the same energy values, and since no energy values are available for crystalline amino acids, the 3 aforementioned diets were assumed to be of equal energy content. This was calculated to be approximately 3,460 kcal metabolizable energy/kg of air-dried diet.

TABLE 1

Composition of crystalline amino acid diet [1]

Ingredient		Amino acid mix	
	%		g/4.2 g
Amino acid mix	4.20	L-leucine	0.29
Cornstarch	79.95	L-methionine	0.10
Cellulose [2]	4.00	L-tryptophan	0.04
Corn oil	6.00	L-valine	0.16
Porcine vitamin mix [3]	1.00	L-lysine·HCl	0.18
Vitamin A and D mix [4]	0.35	L-isoleucine	0.15
Mineral mix no. 2 [5]	4.00	L-phenylalanine	0.10 [6]
$NaHCO_3$	0.50	L-histidine·HCl·H_2O	0.11
Chlortetracycline [7]	+	L-arginine·HCl	0.22
		L-threonine	0.12
Total	100.00	L-glutamic acid	2.73
		Total	4.20

[1] A nitrogen-free diet was exactly the same as above except that an equal weight of starch replaced the amino acid mix.

[2] Solka Floc BW-40, Brown Company, Boston.

[3] Each kg of mix contained the following: (in mg) thiamine·HCl, 260; riboflavin, 660; niacin, 4,400; pantothenic acid, 2,640; pyridoxine, 250; choline chloride, 220,000; vitamin B_{12}, 4.4; menadione, 20; α-tocopherol, 12,000; folic acid, 200; biotin, 20; and ascorbic acid, 30,000. The remainder was finely powdered starch.

[4] Each kg of mix contained 8.5 g vitamin A palmitate and 1.5 g irradiated ergosterol. The remainder was finely powdered starch.

[5] Each kg of mix contained the following: (in grams) $CaHPO_4$, 650.05; NaCl (iodized), 159.96; K_2CO_3, 139.95; $MgCO_3$, 32.74; $FeSO_4 \cdot XH_2O$, 10.00; $MnSO_4 \cdot H_2O$, 3.00; $CoCl_2 \cdot 6H_2O$, 1.00; $CuSO_4$, 1.00; NaF, 0.20; $ZnCO_3$, 2.00; and KI, 0.10.

[6] Phenylalanine was revised from 0.26% to 0.10% of the diet after a preliminary assay revealed that the latter would maximize nitrogen retention.

[7] Crystalline chlortetracycline was added at 20 mg/kg diet.

TABLE 2

Composition of reference diet

	%
Ground yellow corn	14.9
Soybean oil meal (50%)	3.4
Cornstarch	68.3
Cellulose [1]	4.0
Corn oil	6.0
Dicalcium phosphate	2.4
Ground limestone	0.2
Trace-mineralized salt [2]	0.5
Vitamin mix [3]	0.2
Antibiotic [4]	0.1

[1] See table 1.
[2] Contributed the following as a percentage of the ingredient: zinc, 0.800; cobalt, 0.022; manganese, 0.400; copper, 0.048; iron, 0.330; iodine, 0.011; evaporated salt, 97.000; and other, 1.389.
[3] Each kg of the ingredient contained the following: vitamin A and D_3, 3,300,000 and 330,000 USP units, respectively; riboflavin, 1.1 g; Ca pantothenate, 5.5 g; niacin, 16.5 g; choline chloride, 110.3 g; and vitamin B_{12}, 18 mg.
[4] Contained 33 g streptomycin (as sulfate) and 11 g procaine penicillin/kg.

Experiment 1. Eight gilts were used in a completely randomized design to evaluate the reference diet and the nitrogen-free diet at 2 levels of energy intake. The 2 levels were provided by feeding either 1.82 kg/head daily or this amount plus an additional 0.91 kg cornstarch (mixed into the respective diets prior to feeding).

Experiments 2, 3 and 4. The purified, crystalline amino acid diet was used in these experiments. Various amino acids were selected for omission from the diet. Those selected, except for threonine and isoleucine, were amino acids which have been reported as being dispensable for maintenance in one species or another. Whenever an amino acid was removed from the complete amino acid mix it was replaced on an equal nitrogen basis with L-glutamic acid such that all diets fed were isonitrogenous. Eight gilts were used in each of experiments 2, 3 and 4.

Experiment 4 was designed to study nitrogen metabolism of protein-depleted and protein-nondepleted gilts as influenced by a dietary void of arginine, histidine or threonine. Depletion consisted of feeding 1.82 kg/head daily of the nitrogen-free diet for 21 days. Nondepletion consisted of feeding the same quantity of a fortified 16% crude protein corn-soybean meal diet for 21 days. At the end of this pre-metabolism phase (the beginning of the metabolism phase) blood samples (30 to 40 ml) were drawn from the anterior vena cava 2 hours after the morning feeding. At the completion of the metabolism phase, the gilts were similarly bled again. Hemoglobin was determined by the cyanmethemoglobin method described by Crosby et al. (9). Plasma free amino acids were determined by means of an automatic amino acid analyzer (10). Preparation of samples for analysis was carried out according to procedures outlined by Stein and Moore (11), using picric acid to deproteinize the plasma.

Experiment 5. Four gilts were used to evaluate 4 graded levels of leucine ranging from zero to 0.29% of the diet. Two 14-day periods were used such that 2 observations per level (from different gilts) were obtained.

RESULTS AND DISCUSSION

Protein and energy adequacy of reference diet (exp. 1). The results of experiment 1 are presented in tables 3 and 4. The reference diet adequately met the maintenance protein and energy needs of the nongravid gilt. Nitrogen balance was not increased nor was urinary nitrogen decreased by provision of additional carbohydrate calories to gilts consuming either

TABLE 3

Nitrogen metabolism of gilts fed 2 levels of energy intake [1,2] (exp. 1)

Treatment	Mean daily N		
	Urine	Feces	Balance
	g	*g*	*g*
1. 1.82 kg/day reference diet	5.57	2.03	1.40
2. As 1 + 0.91 kg/day cornstarch	6.42	2.57	0.88
3. 1.82 kg/day N-free diet	3.08	1.14	−3.02
4. As 3 + 0.91 kg/day cornstarch	3.41	1.30	−3.01

[1] Eight crossbred gilts — 2/treatment.
[2] Daily N intake was 9.00, 9.87, 1.20 and 1.70 g for treatments 1 through 4, respectively.

TABLE 4

Analysis of variance of nitrogen balance data in table 3

Source of variance	df	Variance
Energy levels (E)	1	0.133
Protein levels (P)	1	34.404*
E × P	1	0.138*
Error	4	0.015

* $P < 0.05$.

the reference diet or the nitrogen-free diet. Since an increase in food intake accompanied the increase in energy intake, fecal nitrogen excretion was greater at the higher level of energy intake. The increase in urinary nitrogen excretion in response to additional calories does not necessarily mean that excess energy from starch decreased the biological value of the dietary protein in the reference diet or enhanced tissue protein catabolism, since some of this increased excretion may have been derived from nonprotein or protein nitrogen present in the starch. Samples of starch were analyzed and found to contain 0.04 to 0.06% nitrogen. For this reason, nitrogen balance is the more appropriate criterion of energy adequacy.

Statistical analysis of the nitrogen balance data revealed a significant response ($P < 0.05$) due to protein. The interaction of protein intake × energy intake was significant ($P < 0.05$), the adverse effect of excess energy on nitrogen balance being substantial for the reference diet but absent for the nitrogen-free diet. Several reports in the literature suggest the opposite; namely, that excess energy will improve nitrogen balance if, and only if, protein is present in the diet (12). However, it could be argued that a response to additional calories should be interpreted to mean that the increment of energy increase represents a change from deficient to less deficient or adequate, instead of from adequate to excess.

As indicated from the urinary nitrogen excretion of gilts consuming the nitrogen-free diet, the endogenous excretion of this element was considerably less than that which would be predicted by the formula of Brody (13). Conversely, the gravid gilt excretes a greater than expected quantity of endogenous nitrogen (7). Since the reference diet used here contained the same pattern of amino acids as that used by Rippel and co-workers (7), a comparison of biological values of the gravid and the nongravid gilt is possible. This results in biological values of 99% and 69% for the gravid and nongravid gilt, respectively. Apparently the gravid gilt, although losing more endogenous nitrogen, has a greater capacity for retaining dietary nitrogen than does the nongravid gilt.

Prior to proceeding with a qualitative evaluation of the amino acid needs of adult swine for maintenance, experiments were conducted to provide information on the effect of a dietary excess of threonine, isoleucine, methionine, phenylalanine, tryptophan, arginine or lysine on nitrogen balance. Excess amino acids were added to the reference diet on an equimolar basis replacing starch, and calculated from threonine which was added at 1% of the diet. No depression of nitrogen balance occurred from any of these additions. Thus, it was concluded that the reference diet (and, hence, the purified, crystalline amino acid diet) would probably not be easily imbalanced by a single amino acid excess in the diet.

Effect of specific dietary amino acid voids (exps. 2, 3 and 4). In order of decreasing effect, a dietary void of threonine, lysine or phenylalanine decreased nitrogen balance (tables 5 and 6), but omission of either arginine or histidine caused no pronounced decrease in nitrogen balance. None of the mammalian species thus far studied has been shown to require arginine for maintenance. It is thought that all mammals actively synthesize this amino acid by a mechanism similar to that of the rat (14, 15). The chicken, however, does not actively synthesize arginine, as evidenced by its relatively high maintenance requirement for this amino acid (16).

Histidine dispensability for maintenance was not unexpected since earlier Rose et al. (17) had demonstrated that man does not require this amino acid. Histidine can be synthesized by man.[4] Perhaps adult swine can synthesize enough histidine to meet their maintenance need.

[4] See footnote 3.

TABLE 5

Nitrogen metabolism of gilts fed diets void of various amino acids [1,2] *(exps. 2 and 3)*

Amino acid omitted	Mean daily N			Relative utilization [3]
	Urine	Feces	Balance	
	g	*g*	*g*	%
None (positive control)	6.73	1.17	1.82	100.0
Arginine (A)	6.61	1.27	1.84	100.4
Histidine (H)	7.20	1.16	1.36	91.4
Phenylalanine (P)	8.58	0.91	0.23	70.4
Lysine (L)	9.29	0.89	−0.46	57.6
Threonine (T)	11.04	1.17	−2.49	19.9
All (negative control)	3.50	1.15	−3.56	0.0

[1] Fourteen crossbred gilts — 2/treatment.

[2] One crossbred gilt was fed 0.50% L-phenylalanine and one an isoleucine-free diet. Their respective N balances were 1.90 and −2.14 g/day.

[3] In terms of N balance $\frac{\text{amino acid void} - \text{negative control}}{\text{positive control} - \text{negative control}} \times 100$.

TABLE 6

Analysis of variance of nitrogen balance data in table 5

Source of variance	df	Variance
Experiment	1	0.130
Voids	6	—
All vs. T, L, P, H, A, none	1	26.007**
T vs. L, P, H, A, none	1	22.767**
L vs. P, H, A, none	1	5.013**
P vs. H, A, none	1	3.110*
H vs. A, none	1	0.298
A vs. none	1	0.001
Error	6	0.272

* $P < 0.05$.
** $P < 0.01$.

A dietary void of phenylalanine (and tyrosine) although causing a lower nitrogen-void balance ($P < 0.05$) than the average of the arginine-void, histidine-void and complete diets, allowed a positive nitrogen balance. However, the observed retention of 0.23 g nitrogen /day is an average value from only 2 gilts — one in slightly negative, the other in slightly positive nitrogen balance. Hence, a final answer as to the phenylalanine need for maintenance cannot be given. It is conceivable that a slow turnover rate of endogenous phenylalanine or tyrosine, or both, could account for the failure herein to obtain negative nitrogen balance with the diet void of aromatic amino acids.

To obtain further evidence that the reduction of phenylalanine from 0.26 to 0.10% of the diet was appropriate, one gilt was fed the positive control diet supplemented with 0.40% phenylalanine. This resulted in a daily nitrogen balance of 1.90 g, not greatly different from 1.82 g observed at the lower level of phenylalanine.

Negative nitrogen balance resulted when lysine or threonine was omitted from the diet. Since Burroughs et al. (18) had implicated isoleucine along with threonine as occupying a key position in maintenance anabolism, one gilt was fed an isoleucine-free diet. This diet effected a negative nitrogen balance which was almost as severe as that observed with the threonine-free diet (table 5, footnote 3), but neither the threonine- nor isoleucine-free diet caused as negative a nitrogen balance as did the nitrogen-free diet.

The observation that diets containing a void of either threonine or isoleucine were very poorly utilized suggests that little if any biosynthesis of these 2 amino acids was being carried out. Data from our laboratory have shown that these 2 amino acids appear to be unique among the classical 10 essential amino acids in that a void of either effects a nitrogen balance much more negative than a void of any of the other amino acids.

The fecal nitrogen data suggest that the digestibility of amino acid nitrogen was 100% regardless of the pattern of amino acids fed. Working with adult rats, Anderson and Nasset (19) observed the same phenomenon.

It is conceivable that the ill-defined and often misundersood "protein stores" could be contributing supplementary endogenous amino acids for protein synthesis. The reports of Bothwell and Williams (20) and

Forbes and Vaughan (21) suggest that histidine may be turned over rather slowly in the animal body. Wissler et al. (22) observed that adult rats fed a histidine-free diet would maintain positive nitrogen balance for one week, but during the second week would be in negative nitrogen balance. These reports imply that a longer feeding period or a prior depletion of "protein stores" may be necessary to manifest a histidine deficiency. To test this hypothesis, and to obtain further evidence on arginine and histidine dispensability for maintenance, diets void of these amino acids were fed to protein-depleted and protein-nondepleted gilts (exp. 4). For comparative purposes, the complete amino acid diet was used as a positive control and the threonine-void diet was used as a negative control.

As before, threonine omission resulted in a marked negative nitrogen balance (table 7) which was significantly different ($P < 0.01$) (table 8) from the average of the other 3 treatments. The nitrogen balance which resulted from feeding the arginine-void diet and the histidine-void diet (pooled observation) was not significantly different from that which resulted from feeding the complete amino acid diet. The nonessentiality of histidine (as well as arginine) for maintenance of the gilt apparently cannot be explained on the basis of a contribution of this amino acid from the "protein stores." No explanation can be offered for the better utilization of a histidine-free diet in this experiment than in the previous experiment. With the exception of the complete amino acid diet, nitrogen balances were more positive (or

TABLE 7

Effect of an arginine, histidine or threonine void on nitrogen balance and hemoglobin of protein-depleted and protein-nondepleted gilts [1,2] *(exp. 4)*

Amino acid omitted	Daily N			Hemoglobin
	Urine	Feces	Balance	
	g	*g*	*g*	*g/100 ml*
	Depleted			
1. None	6.82	1.53	0.95	10.6
2. Arginine (A)	6.53	1.16	1.61	10.0
3. Histidine (H)	5.88	1.09	2.33	12.6
4. Threonine (T)	10.38	1.60	−2.68	10.6
	Nondepleted			
1. None	6.62	1.17	1.51	12.8
2. Arginine	7.49	1.44	0.37	11.8
3. Histidine	5.97	1.21	2.12	13.0
4. Threonine	10.82	1.58	−3.10	13.1

[1] Eight crossbred gilts.
[2] Depleted gilts were fed a N-free diet for 21 days prior to being placed upon the above treatments. The subsequent assay period was 14 days in length with total excreta collection being made on the last 7 days.

TABLE 8

Analysis of variance of data in table 7

Source of variance	df	Variance	
		N balance	Hemoglobin
Depleted vs. nondepleted	1	0.215	5.952*
Voids	3	—	—
T vs. A, H, none	1	28.667**	0.004
None vs. A, H	1	0.190	0.030
A vs. H	1	1.525	3.610
Error (interaction)	3	0.274	0.431

* $P < 0.05$.
** $P < 0.01$.

less negative) in depleted than in nondepleted gilts.

Nassett and Gatewood (23) suggested that the rat may degrade hemoglobin in an attempt to supply endogenous histidine for tissue protein synthesis when the diet is deficient in this amino acid. A slight decrease in hemoglobin occurred during the 14-day metabolism period. However, since this decrease was similar for all treatments, only final values are shown in table 7. While none of the orthogonal treatment comparisons were statistically significant (table 8), depletion versus nondepletion was significant ($P < 0.05$). That hemoglobin was not reduced by feeding a histidine-free diet for 35 days (depleted 21 days with N-free diet, 14 days with histidine-free diet) indicates that in the gilt, hemoglobin was not providing endogenous histidine for usage with other exogenous amino acids for protein synthesis.

Whether gilts were protein-depleted or non-depleted, a dietary void of arginine, histidine or threonine resulted in a large reduction of the same amino acid in the plasma (table 9). In general, protein depletion caused an increase in the concentration of these 3 amino acids and of lysine in plasma when the complete or the arginine-void diet was fed. On the other hand, when a histidine-void diet was fed, the protein-depleted gilt had a lower concentration of arginine, histidine and lysine but a higher concentration of threonine than the nondepleted gilt. Also, a much lower plasma histidine concentration was observed in the protein-depleted gilt consuming a threonine-void diet than in the nondepleted gilt consuming the same diet.

Another observation in connection with the plasma amino acid values was that arginine, histidine and threonine were present in lower concentration when all of the other amino acids were present (void diets) than when none of the other amino acids were present (N-free diet).

Zimmerman [5] showed that when arginine was made growth-limiting for chicks, lysine accumulated in the blood plasma. That lysine did not accumulate in the plasma as a result of feeding an arginine-free diet to gilts could be taken as further evidence that arginine is not essential for maintenance of the adult gilt. Plasma lysine actually decreased (table 9) as a result of feeding an arginine-free diet. Of course, it may be that this lysine-arginine relationship is different for growth than for maintenance.

Leucine assay (exp. 5). Because Burroughs et al. (24) could not obtain a response of adult rats to leucine when it was added to a leucine-void diet, and because Rose et al. (25) could not induce a distinctly negative nitrogen balance in man when leucine was absent from the diet, a qualitative and quantitative evaluation of the leucine need for maintenance was made. Although nitrogen balance increased with increasing levels of leucine, the response was not statistically significant (table 10). Perhaps adult swine can

[5] Zimmerman, R. A. 1965 Plasma amino acid patterns in the chick as affected by suboptimal and superoptimal dietary levels of single amino acids or nitrogen, with special reference to length of feeding period. Ph.D. thesis, University of Illinois.

TABLE 9

Concentration of some amino acids in plasma from protein-depleted (D) and protein-nondepleted (ND) gilts fed diets void of various amino acids [1] (exp. 4)

Plasma amino acids	Amino acid(s) omitted from diet								
	None		Arginine		Histidine		Threonine		All [2]
	D	ND	D	ND	D	ND	D	ND	
	μmole/100 ml		*μmole/100 ml*		*μmole/100 ml*		*μmole/100 ml*		
Arginine	12.7	9.8	5.2	4.1	6.3	10.6	11.7	11.7	11.3
Histidine	4.2	3.4	4.9	4.1	0.6	2.6	1.7	8.6	3.8
Lysine	23.6	17.8	15.4	13.0	8.7	22.8	18.2	16.1	20.7
Threonine	14.5	11.7	10.1	10.9	29.2	20.9	2.8	2.0	13.1

[1] Four depleted (D) gilts were fed a N-free diet for 21 days prior to being placed upon the above treatments. Four nondepleted (ND) gilts received a fortified 16% CP corn-soybean meal diet during the same 21-day period.

[2] Pooled sample from the 4 gilts on the depletion regimen, thus representing the plasma amino acid pattern of gilts fed a N-free diet.

TABLE 10

Nitrogen metabolism of gilts fed graded levels of leucine [1] *(exp. 5)*

Leucine	Mean daily N		
	Urine	Feces	Balance
%	g	g	g
0	7.01	1.53	1.30
0.097	5.69	1.48	2.68
0.193	5.29	1.45	3.10
0.290	5.53	1.39	2.92

[1] Four crossbred gilts were used for two 14-day periods, being allotted to a different treatment after period 1.

synthesize a considerable quantity of this amino acid.

Gilts used in this experiment exhibited more positive nitrogen balances than those observed in previous experiments. For this reason there remains some doubt as to whether leucine is dispensable for maintenance of the nongravid gilt. Also, despite the relatively lengthy experimental period of 14 days, it is possible that an extremely slow turnover rate of endogenous leucine could explain the failure herein to obtain a graded response. However, since an average balance of over 1 g/day resulted from a diet completely devoid of this amino acid, it must be tentatively considered dispensable.

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Quantitative Evaluation of the Threonine, Isoleucine, Valine and Phenylalanine Needs of Adult Swine for Maintenance

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ABSTRACT A completely purified diet containing crystalline L-amino acids as the sole source of dietary nitrogen was used to evaluate the threonine, isoleucine, valine and phenylalanine requirements for maintenance of the nongravid gilt. To determine the requirement for individual amino acids a quadratic regression equation was fitted to the nitrogen balance data and used to determine the amino acid intake corresponding to a nitrogen balance of 1 g/day (maintenance requirement) and zero (nitrogen equilibrium requirement). Expressed as mg/day/kg$^{3/4}$, requirements for maintenance of 39, 30, 21, and 21 were found for threonine, isoleucine, valine and phenylalanine, respectively. Distinctly negative nitrogen balance resulted from a dietary void of threonine, isoleucine or valine, but a dietary void of phenylalanine (and tyrosine), although causing a reduced nitrogen balance as compared with the complete amino acid diet, did not effect negative nitrogen balance. The excretion of urea plus ammonia in the urine was directly related to the quality of the amino acid pattern fed. The urinary nitrogen differential between a deficient and an adequate level of threonine, isoleucine or valine was represented largely by urea plus ammonia. Whether suboptimal or superoptimal, the concentration of valine in the diet determined the concentration of free valine in the blood plasma.

Maintenance can be defined as a stable state of equilibrium among the different but interdependent elements and systems of an organism. Difficult as it may be to prove the presence of this condition, much work has been carried out on the maintenance requirements of the small laboratory animal. However, other than for the adult human, little information is available on the maintenance needs, particularly for amino acids, of large animal species. Until recently, the cost of such experimentation has been prohibitive. Furthermore, inadequate consumption of purified amino acid diets, based primarily on bland, synthetic ingredients, has been a major obstacle to the concise interpretation of the data reported. In this respect the porcine, by reason of its voracity, appears ideally suited for a study of this type.

In a previous paper (1) a qualitative evaluation of the amino acid needs of the adult, nongravid gilt for maintenance was presented. These data indicated that of the classical 10 essential amino acids, three, arginine, histidine and leucine, were not required for maintenance. The work presented herein is an attempt to quantify the requirements for threonine, isoleucine, valine and phenylalanine, using nitrogen retention as the primary criterion of adequacy.

EXPERIMENTAL PROCEDURE

Selection and feeding of gilts as well as collection procedures were similar to those described previously (1). All gilts used in this series of experiments were crossbreds weighing approximately 145 kg. The basal, crystalline amino acid diet is shown in table 1. Even though arginine, histidine and leucine were found to be dispensable for maintenance they were included because some of the qualitative data had not been analyzed prior to starting the quantitative phase of these experiments; and also it was felt that removal of arginine, histidine and leucine from the amino acid mix might create an unfavorable indispensable-to-dispensable amino acid ratio.

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[2] This paper represents part of a thesis submitted by the senior author to the Graduate College of the University of Illinois, in partial fulfillment of the requirements for the Ph.D. degree.

Gilts were fed 1.82 kg/head daily and all diets were maintained isonitrogenous both between and within assays by adjusting the level of glutamic acid. Other than cornstarch, which was used to bring all diets up to 100%, and glutamic acid, the amino acid under study in a given assay was the only dietary variable.

In addition to evaluating nitrogen retention, urinary urea plus ammonia nitrogen was determined according to procedures outlined by Fawcett and Scott (2). This method allowed quantitative measurement of both the ammonia produced by urease acting on urea and any preformed ammonia which was present in the sample. Originally, quantitative determination of both urea and ammonia was performed simultaneously, substituting an equal volume of distilled water for urease in the determination of preformed ammonia. However, it soon became evident that the preformed ammonia concentration of the urine samples was so low that precise measurement could not be accomplished without altering the dilution procedures. Therefore, it was decided not to differentiate between these 2 sources of ammonia, but instead to measure total ammonia from both sources.

When applicable, the maintenance requirements for individual amino acids were determined by fitting a quadratic regression equation ($Y = a+bx+cx^2$) to the data and determining the amino acid intake corresponding to a daily nitrogen balance of 1 g (adopted maintenance requirement) and zero (nitrogen equilibrium requirement). The data were not fitted as well by a linear or an asymptotic model as by a quadratic model.

A completely randomized design was used to assay the requirement for isoleucine; a randomized complete block design was used for theonine and valine. The basis for forming blocks in the latter design was the premetabolism feeding regimen and the environmental temperature to which gilts had been subjected.

The concentration of free valine in blood plasma of gilts consuming graded levels of valine was also evaluated. Bleeding procedures and plasma analysis were similar to those described previously (1).

RESULTS

Threonine assay. Significant ($P < 0.01$) linear and quadratic effects were found in the nitrogen balance data resulting from feeding graded levels of threonine (table 2). Substituting Y values of 1 and zero grams nitrogen balance per day into the fitted quadratic regression equation ($Y = -2.454+54.275X-172.917X^2$) yielded requirements of 0.089% (39 mg/day/$kg^{3/4}$) and 0.055% (24 mg/day/$kg^{3/4}$) dietary threonine for maintenance and nitrogen equilibrium, respectively.

A significant difference ($P < 0.05$) between replicates was observed. Replicate 1 consisted of 4 gilts which had been used in a previous assay and had thus been

TABLE 1

Composition of crystalline amino acid diet [1]

Ingredient		Amino acid mix	
	%		g/4.2 g
Amino acid mix	4.20	L-leucine	0.29
Cornstarch	79.95	L-methionine	0.10
Cellulose [2]	4.00	L-tryptophan	0.04
Corn oil	6.00	L-valine	0.16
Porcine vitamin mix [3]	1.00	L-lysine·HCl	0.18
Vitamin A and D mix [3]	0.35	L-isoleucine	0.15
Mineral mix no. 2 [3]	4.00	L-phenylalanine	0.10
$NaHCO_3$	0.50	L-histidine·HCl·H_2O	0.11
Chlortetracycline [4]	+	L-arginine·HCl	0.22
		L-threonine	0.12
Total	100.00	L-glutamic acid	2.73
		Total	4.20

[1] Contained approximately 3,460 kcal metabolizable energy/kg of air-dried diet.
[2] Solka Floc BW-40, Brown Company, Boston.
[3] Baker et al. (1).
[4] Crystalline chlortetracycline was added at 20 mg/kg diet.

confined to the metabolism crates for a period of 21 days prior to initiating collection. Noteworthy also is that these gilts had been consuming maintenance-type diets (1.82 kg/head/day) during this entire 21-day period. Replicate 2 consisted of 4 gilts which were selected from the general herd. They had been consuming 1.82 kg/head/day of a fortified 16% crude protein corn-soybean meal diet prior to being placed in the metabolism crates. Therefore, with respect to prior feeding regimen, gilts in replicate 1 had, in essence, a 21-day pretest feeding period, but gilts in replicate 2 had only the normal 7-day pretest feeding period.

Another difference between replicates should be emphasized. Since this assay was conducted during the coldest part of the winter months, gilts of replicate 2 had been subjected to a much colder prior environmental temperature than gilts of replicate 1.

Urinary urea-plus-ammonia nitrogen excretion decreased as dietary threonine increased up to 0.12%. This suggests that a dietary level of 0.12% threonine or slightly more was adequate in this particular diet to maximize exogenous nitrogen utilization.

Isoleucine assay. Each increment of isoleucine caused an improvement in nitrogen balance (table 3). An extremely negative nitrogen balance was exhibited by gilts consuming the diet devoid of isoleucine. Significant ($P < 0.01$) linear, quadratic and cubic effects were found in nitrogen balance. A quadratic regression equation ($Y = -2.855 + 77.100X - 308.480X^2$) indicated that 0.069% (30 mg/day/$kg^{3/4}$)

TABLE 2

Nitrogen metabolism of gilts fed graded levels of threonine [1]

Threonine	Daily N [2]			
	Urine		Feces	Balance
	Total	Urea + NH_3		
%	g	g	g	g
	Replicate 1			
0	10.13	6.37	1.08	−1.90
0.06	7.86	4.67	1.00	0.45
0.12	5.99	3.14	1.34	1.98
0.18	5.54	3.12	1.69	2.08
	Replicate 2			
0	11.07	7.55	1.18	−2.94
0.06	8.53	6.08	1.07	−0.29
0.12	6.84	4.31	1.12	1.35
0.18	6.36 [3]	4.37 [3]	1.34	1.61

[1] Eight crossbred gilts.
[2] Daily N intake was 9.31 g.
[3] Missing value calculated.

TABLE 3

Nitrogen metabolism of gilts fed graded levels of isoleucine [1]

Isoleucine	Mean daily N [2]			
	Urine		Feces	Balance
	Total	Urea + NH_3		
%	g	g	g	g
0	10.76	7.73	1.73	−3.01
0.05	7.42	4.25	1.36	0.70
0.10	6.63	3.56	1.56	1.29
0.15	6.42	3.32	1.13	1.93

[1] Eight crossbred gilts that had been used in the previous (threonine) assay — 2/treatment.
[2] Daily N intake was 9.48 g.

dietary isoleucine was required for maintenance and 0.045% (20 mg/day/kg$^{3/4}$) for nitrogen equilibrium.

Urinary nitrogen derived from urea and ammonia decreased in a manner corresponding to the decrease in total urinary nitrogen, as the dietary level of isoleucine was increased.

Valine assay. Although gilts (replicate 1) that had been used in the previous (isoleucine) assay tended to exhibit more positive nitrogen balances than those (replicate 2) selected from the general herd, the difference between replicates was not statistically significant (table 4). However, this assay was conducted during a warmer season and thus the premetabolism environmental temperature differential between replicates was less than in the threonine assay.

One gilt failed to consume its daily diet allotment of 1.82 kg and appeared sluggish. The gilt's temperature on day 3 of the collection period was 40°, indicating fever and possible infection. Urine analysis revealed an unusually high nitrogen excretion (10.09 g/day) for the treatment (0.12% valine), and data for this animal were omitted from the statistical analysis.

A significant ($P < 0.05$) linear response to valine occurred. A quadratic regression equation ($Y = -1.435 + 62.890X - 258.670X^2$) was calculated and used to obtain the valine requirement for maintenance and nitrogen equilibrium, although the quadratic regression coefficient approached but did not reach the 5% significance level. These were 0.048% (21 mg/day/kg$^{3/4}$) and 0.026% (11 mg/day/kg$^{3/4}$) dietary valine, respectively.

As in the threonine and isoleucine assays, the excretion of urea plus ammonia in the urine closely followed the excretion of total nitrogen in the urine, indicating that both criteria were descriptive of and sensitive to the dietary quality of amino acids fed. The urinary nitrogen differential between a deficient and an adequate level of threonine, isoleucine or valine was represented almost solely by urea plus ammonia, the principal components of the exogenous nitrogen catabolism.

Plasma free valine concentration was found to be directly related to the dietary concentration of valine. Apparently a plasma valine concentration of 5.3 μmole/100 ml plasma is compatible with maintenance of the nitrogenous integrity of the body under conditions imposed by this assay.

Phenylalanine. Quantitative evaluation of the maintenance need for this amino acid was not made since it was felt that the expected range of response (0.23 to 1.82 g nitrogen retention/day based upon the qualitative data (1) from our laboratory) would not be sufficient to yield a useful dose-response curve. Instead, the aforementioned mean nitrogen balances obtained at zero and 0.10% dietary phenylalanine were connected by a straight line, and the intersect of this line with a daily nitrogen balance of 1 g was determined. This yielded a requirement value of 0.049% (21 mg/day/kg$^{3/4}$) dietary phenylalanine for maintenance. The retention of 1.82 g nitrogen/day at 0.10% dietary phenylalanine represents a point somewhere on the plateau of the theoretical dosage response curve. Thus, the indi-

TABLE 4

Nitrogen metabolism of gilts fed graded levels of valine [1]

Valine	Mean daily N [2]				Plasma free valine
	Urine		Feces	Balance	
	Total	Urea + NH_3			
%	g	g	g	g	μmole/100 ml
0	10.33	7.30	1.34	−1.42	2.3
0.06	7.55	4.76	1.37	1.33	5.3
0.12	6.85	4.32	1.25	2.15	14.6
0.18	7.14	4.32	1.63	1.48	20.3

[1] Seven crossbred gilts — 1 gilt on the 0.12% valine treatment became ill and was removed from the experiment; otherwise, all data represent an average of 2 gilts.
[2] Daily N intake was 10.25 g.

cated requirement value is an overestimate, but probably only slightly so, because previous data indicate that 0.10% phenylalanine comes close to the minimum for maximal nitrogen retention.

Two gilts were fed a diet containing no phenylalanine but 0.10% tyrosine. The average nitrogen balance for this treatment was − 0.17 g/day, suggesting that tyrosine had very little, if any, replacement value for phenylalanine. This observation is in contrast to the work of Rose and Wixom (3) who reported that tyrosine was capable of exerting a sparing effect of 70 to 75% upon the phenylalanine requirement of man. However, the possibility exists, as in the human studies, that a tyrosine-sparing effect would have been noted if studied in the presence of phenylalanine.

DISCUSSION

Pertinent to the determination of an amount of any nutrient required for maintenance is the selection of a suitable criterion. The majority of investigators dealing with this problem have used nitrogen equilibrium as the value taken to be commensurate with maintenance. Yet, nitrogen equilibrium calculated from nitrogen balance experiments is not true equilibrium. This is so because the method of nitrogen balance includes only nitrogen losses via the feces and urine and therefore completely ignores nitrogen losses of the integuments. The latter can amount to a considerable daily loss since these structures (epidermis, hair, nails, wool, feathers, etc.) have a rapid turnover and such losses involve highly nitrogenous keratins.

In addition to the nitrogenous losses which are ignored, a positive cumulative error in the nitrogen balance technique, as pointed out by Wallace (4), also makes questionable the validity of nitrogen equilibrium by the method of nitrogen balance as a reference point of amino acid adequacy. Thus, the numerical value obtained for intake is always larger than actuality and the numerical value for output by way of feces and urine is always smaller, since a certain quantity of both the measured intake and the measured excreta is lost. These errors are additive and can therefore lead to erroneously elevated values of nitrogen retention. When amino acid requirements are defined in terms of a particular degree of nitrogen balance sought, these technical errors lead to an underestimation of the true requirements.

Because of the above considerations, Hegsted (5) suggested that a positive nitrogen balance of 0.5 g/day might be compatible with true equilibrium in man. Mitchell (6) and Mitchell and Edman (7) felt that 1 g/day would be closer to true equilibrium in the same species. In the work presented herein, the maintenance requirement was arbitrarily defined as that level of an amino acid which would effect a daily nitrogen retention of 1 g when this particular amino acid was the only dietary variable.

It was noteworthy that a statistically significant replicate effect was obtained in the threonine assay. Since prior feeding regimen and prior environmental temperature were known differences between replicates, one or both of these might be implicated as causative. A general observation has been that gilts on a higher plane of nutrition and exposed to a lower environmental temperature exhibit less positive nitrogen balances in subsequent nitrogen balance trials. Although gilts of replicate 2 had received a pre-assay diet considerably higher in protein (approximately equal in energy and other nutrients) than gilts of replicate 1, the 7-day pretest feeding period appears to be sufficient to eliminate much of the nutritional effect. The environmental effect appears to be the more logical one.

Working with adult rats Treichler and Mitchell (8) observed that a prior low environmental temperature elevated the endogenous loss of nitrogen subsequently determined. The effect of temperature on nitrogen excretion was greater than that of prior plane of nutrition. Issekutz et al. (9) observed the same results in their work with man. The action of cold on protein metabolism may be manifested by an increase in thyroid and adrenal cortex activity (10, 11), both of which would cause an impairment of nitrogen balance.

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Methionine and Cystine Requirements of the Young Guinea Pig [1]

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ABSTRACT The methionine and cystine requirements of the young guinea pig were determined from the 6-week growth response. The basal diet contained 20% of isolated soy protein with various amino acids added to levels equivalent to those in 30% soy protein. Without supplemental methionine, 0.24% of added L-cystine was required. With adequate cystine, 0.15% of added L-methionine was optimal. The total sulfur-containing amino acid requirement was 0.71% (0.36% from cystine and 0.35% from methionine). D-Methionine was not as active as the L-isomer and appeared to be slightly inhibitory. The hydroxy analogue of methionine was less active than D- or L-methionine. Liver weight was markedly affected by the dietary level of methionine; testes and spleen also responded positively, but kidneys and adrenals were unaffected.

In a continuation of studies on the nutritional requirements of the guinea pig, the methionine and cystine requirements and the effects of sulfur-containing amino acid deficiency have been investigated. Because of the work with the rat (1) and the chick (2) it has been assumed for many years that the D- and L-isomers of methionine have equal biological activity. The present results show that this is not true in the guinea pig.

METHODS

Male guinea pigs (Hartley strain) 3 to 5 days old, weighing 95 to 115 g were fed a modification of our semi-purified guinea pig diet (3). The ration contained the following: (in per cent) purified soybean protein,[2] 20; corn oil, 7.3; sucrose, 10.3; powdered cellulose,[3] 15; cornstarch, 20; glucose, 17.8; potassium acetate, 2.5; magnesium oxide, 0.5; salts A (4) in the early studies and salts N (5) in the later tests, 6.0; choline chloride, 0.2; inositol, 0.2; ascorbic acid, 0.2; and liberal amounts of the known vitamins as previously used. The 0.1% level of L-methionine in soy protein, as reported by the producer, was verified by microbiological assays for methionine. Previous studies with this diet (6, 7) had shown that growth was slow with a 20% level of soy protein and that the chief deficiency was methionine. Tryptophan was the only other essential amino acid which might be insufficient to allow maximal growth. To insure against the possibility of such an inadequacy, an addition of 0.1% of L-tryptophan was made. Diets prepared with the 30% level of soy protein contained only 10% starch. In most of the experiments from 5 to 10 animals were started on the diet in each group. The diets were refrigerated at 5° until used and were prepared in amounts to last no longer than 2 weeks.

RESULTS

Animals fed the diet prepared with a 20% level of the soy protein and no added methionine grew slowly. The methionine contained in this level of soy protein amounted to approximately 0.2% of the diet and the cystine to 0.12%. The effect of this diet on the growth rate is shown in the data of table 1. In the total of 17 animals comprising this group, 2 deaths occurred before the end of the sixth week. Other than a poor appetite and slow growth, no specific characteristics were observed. The few blood studies made did not give definite indication of anemia as determined by the hemoglobin level and erythrocyte count. Further tests would be

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[1] Paper no. XII in the series, "Nutritional Studies with the Guinea Pig."

[2] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Cincinnati.

[3] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.

TABLE 1

Growth of guinea pigs fed diets containing 20 or 30% soybean protein with supplementary methionine

Soy protein	Methionine	No. of experiments	No. of animals	Weeks		
				2	4	6
%	%			g	g	g
20	None	4	17	127 ± 26 [1]	198 ± 26	266 ± 34
	0.5 DL-	2	14	133 ± 9	221 ± 17	307 ± 31
	0.75 DL-	3	18	149 ± 13	241 ± 20	336 ± 33
	1.00 DL-	2	14	152 ± 14	253 ± 17	354 ± 31
	2.00 DL-	1	5	128 ± 11	221 ± 24	351 ± 29
	0.375 L-	7	32	154 ± 17	252 ± 30	363 ± 44
	0.375 D-	7	35	143 ± 17	258 ± 37	321 ± 35
	0.425 MHA [2]	3	15	143 ± 12	236 ± 24 [3]	—
30	None	3	28	153 ± 16	260 ± 23	353 ± 34
	0.50 DL-	2	14	181 ± 14	286 ± 25	382 ± 37
	1.00 DL-	1	5	182 ± 22	289 ± 36	391 ± 44

[1] Means ± SD.
[2] Calcium salt of α-hydroxy-γ-methylhydroxybutyric acid, Monsanto Chemical Company, St. Louis.
[3] Experiment terminated after 4 weeks.

desirable, however, before concluding absence of any effect of methionine deficiency on the hemopoietic system in the guinea pig.

With the addition of 0.5% of DL-methionine to the diet, growth was considerably improved ($P < 0.01$; Student's t-test) and no deaths occurred. Raising the level of added DL-methionine to 0.75% resulted in a further significant increase in growth ($P < 0.01$). The average weight at the end of 6 weeks of 336 ± 33 g may be considered as close to maximal for a 20% soy protein diet. In previous studies with soy protein diets it has been shown (7) that with a 20% protein diet supplemented with 1.0% DL-methionine the maximal weight achieved was approximately 368 ± 23 g. With the protein level raised to 30% and supplemented with 0.5% DL-methionine, the maximal weight was raised to 394 ± 37 g. In another study (6) with a 40% level of protein and no methionine supplement, a maximal weight of 400 g was achieved. The addition of 1.0 or 2.0% DL-methionine appeared to produce a further slight increase in growth; but the differences are not significant. At the 2.0% level there appeared to be a slight retardation in growth during the first 4 weeks, but between the fourth and sixth week, appetite for the diet appeared to improve and growth became maximal. No definite differences in results were observed with the 2 salt mixtures.

With the 30% level of soy protein in the diet, an excellent rate of growth and 100% survival was obtained without the addition of methionine; but a small increase in growth ($P < 0.01$) occurred with the addition of 0.5 or 1.0% of DL-methionine. These results showing the effect of supplementing 20 and 30% soy protein diets with methionine are in close agreement with observations reported previously (7).

Comparative values of optical isomers of methionine for growth

Since close to maximal growth was achieved with the addition of 0.75% of DL-methionine to the 20% soy protein diet, tests were made with additions of 0.375% levels of L- and D-isomers (table 1). This level of the L-isomer produced maximal or close to maximal growth. With addition of the D-form, growth was definitely retarded in three of the 7 experiments. The average body weight after 6 weeks of the group receiving D-methionine was 321 g as compared with 363 g for the animals receiving the L-form. In one test in which the D-methionine supplement was increased threefold, the average body weight at the end of 4 weeks equaled that of the group receiving 0.375% of L-methionine. No definite reason can be given for the variations in response to the 2 isomers in different experiments. There was some in-

TABLE 2

Organ weights of guinea pigs fed diets containing 20 or 30% of soy protein with methionine supplements [1]

Soy protein	Methionine	Organ weights					Ratio of liver wt to body wt
		Kidneys	Spleen	Testes	Adrenals	Liver	
%	%	*g*	*g*	*g*	*g*	*g*	
20	None	2.48 ± 0.33 [2]	0.306 ± 0.07	0.353 ± 0.22	0.120 ± 0.02	12.78 ± 2.3	4.80 ± 0.64
	0.5 DL-	2.84 ± 0.42	0.362 ± 0.07	0.497 ± 0.11	0.133 ± 0.02	16.49 ± 4.0	5.37 ± 0.91
	0.75 DL-	3.09 ± 0.37	0.465 ± 0.11	0.590 ± 0.13	0.150 ± 0.02	19.44 ± 3.8	5.80 ± 0.88
	1.00 DL-	3.59 ± 0.38	0.527 ± 0.06	0.623 ± 0.10	0.146 ± 0.03	21.05 ± 3.4	5.95 ± 0.90
	0.375 L-	3.23 ± 0.33	0.606 ± 0.15	0.717 ± 0.12	0.143 ± 0.02	25.36 ± 4.4	6.00 ± 1.26
	0.375 D-	3.10 ± 0.30	0.405 ± 0.09	0.547 ± 0.15	0.139 ± 0.02	17.42 ± 2.9	5.42 ± 0.70
30	None	3.54 ± 0.38	0.415 ± 0.03	0.63 ± 0.09	0.141 ± 0.02	21.75 ± 3.7	6.02 ± 0.74
	0.5 DL-	3.74 ± 0.42	0.527 ± 0.17	0.827 ± 0.14	0.157 ± 0.02	24.56 ± 4.5	6.42 ± 0.77
	1.0 DL-	3.77 ± 0.51	0.642 ± 0.14	0.795 ± 0.10	0.152 ± 0.02	24.57 ± 2.4	6.37 ± 0.78

[1] Same animals as in table 1.
[2] Means ± SD.

dication of a difference in digestive function between the D- and L-methionine groups as shown by the fact that the weight of the digestive tract with contents of the D-methionine animals was 97 g as compared with 85 g in the L-methionine group. The former constituted 30% of the body weight, whereas in the latter it amounted to 24%. The animals reared with the diet without added methionine had the same gastrointestinal-to-body weight percentage values as did the D-methionine group.

That better growth was achieved with the addition of 0.375% of the L-isomer than with 0.75% of the DL-compound suggests that a slight inhibitory effect may result from the presence of the D-isomer.

Variations in organ weights with changes in the amounts and types of dietary methionine

Effect of protein level: The effect of 20 and 30% levels of soy protein with and without added methionine on the weights of some of the internal organs is shown in the data on table 2. This table also shows the relation of liver weight to body weight. The addition of methionine had a marked effect on the liver. With a 20% level of protein and no added methionine, none of the livers of the 15 animals attained a weight of 20 g, only three of them weighed over 15 g, and three were under 10 g. With the 30% level of protein, only five had liver weights below 20 g and two had weights above 25 g. The *P* value for the difference in liver weights of the 20 and 30% dietary protein groups was < 0.025. Very striking differences in liver weights occurred between animals receiving supplements of L- and D-methionine. Organ weights were obtained on 18 animals of each of these groups. With D-methionine, only 4 livers had weights above 20 g, none were above 25 g and one was under 15 g. In the group receiving L-methionine, only two had livers weighing less than 20 g, 8 had weights above 25 g, three were above 30 g, one weighed 38 g and another one 43 g.[4] These livers appeared normal in color and texture and showed no gross evidence of fattiness. The *P* value for the

[4] This animal was extremely vigorous and healthy. It weighed 433 g at 6 weeks.

difference in liver weights between the L- and D-methionine groups was < 0.001.

Comparative value of methionine and hydroxy-methionine analogue

When the calcium salt of α-hydroxy-γ-methylhydroxybutyric acid [5] (MHA) was substituted for methionine on an equimolar basis, growth was significantly improved over that in the basal group. However, the average weight at 4 weeks was slightly inferior to that obtained with D- or L-methionine, although the differences probably are not significant (table 1). It was apparent that the diet with MHA was less acceptable, with a consequent decrease in food intake. This compound is apparently not a satisfactory substitute for methionine in the guinea pig. In the chick, Gordon and Sizer (8) observed no difference between the response to MHA and L-methionine when the 2 compounds were fed on an equimolar basis. D-Methionine was found to be not fully equivalent to the other two. Colet and Baratou (9) also compared the growth efficiency for chicks of L-methionine and MHA and reported that methionine was slightly superior as to gain in weight, but was always inferior in the food consumption index. Tipton et al. (10), in tests with chicks, observed that DL-methionine consistently supported better growth than did MHA when comparisons were made either on an equimolar or equal weight basis.

Methionine requirement of the guinea pig in relation to other amino acids

Although the addition of 1.0% of DL-methionine to the 20% soy protein diet resulted in a marked gain in weight (266 g compared with 354 g at 6 weeks) the increase did not equal that achieved with the 30% soy protein diet also supplemented with 1.0% DL-methionine (391 g at 6 weeks). It was evident that at the 30% protein level the requirement of amino acids other than methionine was better satisfied. It seemed possible that an insufficiency of cystine at the 20% protein level could have been partially responsible for the difference. Tests were accordingly conducted to determine to what extent the inadequacy could have been due to cystine or to other amino acids, or to a combination of cystine and other amino acids. Additions of the following amino acids supplied (as g/kg of diet) were made to the 20% protein diet to bring the respective levels up to that in the 30% level of protein: glycine, 2.40; L-leucine, 3.60; L-threonine, 1.40; L-tryptophan 1.10; L-phenylalanine, 1.70; L-valine, 1.50. Diets containing 20% soy protein and the amino acid mixture were prepared and to different lots of diet L-cystine was added in amounts ranging from none to 3.6 g/kg. Additional glycine was added in amounts sufficient to make the diets approximately isonitrogenous. The experimental conditions for the 4 groups of animals and the results are shown in table 3. The addition of amino acids other than cystine produced a considerable improvement in growth and survival with the average weight at 4 weeks of 218 ± 21 g as compared with 198 ± 26 g without the addition of the amino acid mixture (table 1). The addition of L-cystine led to further increases with the maximal effect occurring with the

[5] Monsanto Chemical Company, St. Louis.

TABLE 3

Growth of guinea pigs fed diets containing 20% soy protein with supplementary cystine [1]

Cystine	Glycine	No. of experiments	No. of animals	Weeks			
				1	2	3	4
g/kg	*g/kg*			*g*	*g*	*g*	*g*
None	2.25	4	20	115 ± 7 [2]	143 ± 11	174 ± 16	218 ± 21
1.20	1.50	4	20	128 ± 14	158 ± 20	205 ± 16	243 ± 21
2.40	0.25	3	15	128 ± 9	170 ± 17	219 ± 19	259 ± 22
3.60	0.0	2	10	131 ± 13	171 ± 19	213 ± 20	256 ± 26

[1] Basal diet contained a mixture of amino acids to make it isonitrogenous with a 30% protein level (see text).
[2] Means ± SD.

supplying of 2.4 g/kg. At this level the average weight after 4 weeks was 259 ± 22 g as compared with 260 g for a 30% level of protein (table 1).

Methionine requirement with ample cystine

Further studies were next made to determine the methionine requirement with adequate dietary cystine. Except for methionine, the dietary content of essential amino acids was approximately the same as would be found in a 30% level of the soy protein. To the 20% basal diet were added the amino acid mixture (11.7 g/kg) and L-cystine (2.4 g/kg). Three experiments were conducted with 15 animals each. The added L-methionine of the diet ranged from none to 0.45% of the diet. Results of the tests are shown in table 4. Maximal growth was achieved with the addition of only 0.15% of L-methionine. The weight after 6 weeks (385 ± 45 g) was the same as that achieved with a 30% level of the protein supplemented with 0.5% of DL-methionine (382 ± 37 g, table 1), but was greater than that obtained with the unsupplemented protein (353 ± 34 g). The difference in weight is due to the fact that at the 30% level of the soy protein, the diet contains an inadequate amount of both methionine (0.3%) and cystine (0.18%) to support maximal growth. There was some evidence from the survival data that the highest level of added methionine (0.45%) may have had an unfavorable effect during the later phase of the growth period.

TABLE 4

L-Methionine requirement with adequate dietary cystine [1]

L-Methionine	Weeks		
	2	4	6
%	g	g	g
None	165 ± 29	256 ± 31	300 ± 41
0.15	181 ± 32	273 ± 32	385 ± 45
0.25	196 ± 21	279 ± 26	368 ± 46
0.35	188 ± 20	284 ± 33	393 ± 32
0.45	191 ± 25	273 ± 43	373 ± 38

[1] Average of 3 experiments, 15 animals/diet.

DISCUSSION

The sulfur-containing amino acid requirement of the guinea pig fed a 20% soy protein diet supplemented with adequate methionine and cystine would be 0.71% of the diet with 0.36% being furnished as L-cystine and 0.35% as L-methionine. The requirement for sulfur-containing amino acids thus appears to be less than that of the chick, which is 0.8% of the diet of which 0.45% of this amount must be methionine (11).

The requirement of the guinea pig appears to be slightly higher than that of the rat (0.6%, of which one-third to one-half may be cystine) (12), and much higher than that of the cat (0.12%).[6]

The present results indicate that the liver of the guinea pig is quite sensitive to changes in the methionine content of the diet and suggest that changes in liver weight may be a better criterion of the methionine requirement than are changes in body weight. In the rat it has been shown that the liver takes priority for the methionine supply in time of deficiency (13). Kertai and Sós (14) studied the uptake of methionine-^{35}S in rats fed a methionine-deficient diet and found that methionine was first taken up by the liver and brain. Edwards et al. (15) observed that the rate of absorption of L-methionine was 1.1 times that of the D-isomer. Edwards and Gadsen [7] reported that protein synthesis by the liver, thymus, and seminal vesicles was greater with the L-isomer. From other studies with the rat (16–18) considerable knowledge has accumulated which indicates that methionine deficiency produces definite effects upon some, but not all, of the liver enzyme systems with little or no effect on endogenous respiration of liver tissue in vitro.

Fell et al. (19) and Bauridel (20) reported that D- and L-methionine are equally effective for growth in the chick. Marrett et al. (21) observed however, that the chick's ability to utilize D-methionine is influenced to a very great extent by the level of other D-amino acids in the basal ration. With a diet containing 15% D-amino acids (3.8% of basal diet) L-methionine was utilized better than the D-form.

[6] Dymsza, H. A., and S. A. Miller 1964 Dietary methionine requirement of the cat. Federation Proc., 23: 512 (abstract).

[7] Edwards, C. H., E. L. Gadsen, C. Higgenbotham and G. A. Edwards 1964 Utilization of D- and L-methionine for protein synthesis. Federation Proc., 23: 511 (abstract).

With an all L-amino acid basal mixture the 2 forms of methionine were utilized equally well. Bauer and Berg (22) reported that the 2 forms of methionine were utilized similarly by the mouse. The observation of a slightly faster rate of absorption of the L-isomer in the rat (15) may not upset the conclusion of Wretland and Rose (23) that the 2 isomers are equally valuable for growth in the rat.

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Effects of Magnesium, Potassium and Sodium Nutriture on Mineral Composition of Selected Tissues of the Albino Rat

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ABSTRACT A total of 196 growing albino rats was used in 3 experiments to investigate the effects of deficient and moderately excessive supplies of Mg, K and Na on concentration of these minerals and Ca in selected tissues. The data support the previously reported view that Mg deficiency induces as a secondary effect many of the changes associated with K deficiency, i.e., a decrease in serum, skeletal muscle, kidney and bone K and a compensatory increase in skeletal muscle and heart Na. The only common effects of Mg and Na deficiencies were decreases in kidney and bone Na and in kidney Mg. Both Na and K deficiencies increased serum and bone Mg. Moderate excesses of K and Na as chlorides did not significantly affect tissue mineral composition, but when supplied as carbonates both minerals increased serum K, decreased serum Mg of animals fed a Mg-deficient diet, and bone Mg of animals fed a Mg-adequate diet. Potassium, but not Na carbonate, lowered serum Mg of animals fed adequate Mg. The most striking effect of both carbonates was that of increasing kidney calcification of Mg-deficient animals.

The development of potassium deficiency, as evidenced by decrease in muscle potassium, in animals receiving potassium-adequate, magnesium-deficient diets has been documented previously (1, 2). It has been demonstrated more recently that increasing the dietary level of potassium does not prevent this secondary potassium depletion (3, 4). In these latter reports the positive control diets contained potassium and magnesium well above the requirement levels for the rat.

It was an objective of the current investigation to study further the effects of graded levels of supplementary magnesium and of potassium on weight gain and tissue mineral composition in an attempt to differentiate the effects of a direct potassium deficiency and those produced secondary to magnesium deficiency. We were also interested in the effects of sodium supply on tissue composition under varied states of magnesium nutriture in view of the observation (3, 4) that potassium and magnesium deficiencies are accompanied by increase of skeletal muscle sodium as the potassium decreases. Finally, we investigated the effects of supplying excess sodium and potassium as carbonates rather than as chlorides as was done in the first experiment, thus providing a change in the cation-anion ratio of the diet. Cotlove et al. (1) have reported that alkalosis, produced by intraperitoneal flushing with $NaHCO_3$ to deplete the tissues of chloride decreased serum and muscle K and increased muscle Na.

METHODS

In the 3 experiments to be described certain procedures were common to all. Weanling male albino rats of Sprague-Dawley strain were used. They were individually housed and fed ad libitum the respective diets for 28 days. Deionized water was always available to them. Feed intake records were kept and body weights were recorded at weekly intervals. At the end of the experiments the animals were decapitated and samples of the tissues to be analyzed were obtained.

The basal diet contained 15% casein, 0.3% DL-methionine, 8.0% corn oil, 0.50% cod liver oil, 3.0% cellulose, 5.0% vitamin glucose mixture (5), 2.40% mineral mixture and 65.8% glucose monohydrate. The basal mineral mixture in experiment 1 consisted of reagent grade chemicals in the following percentages of the mixture: $CaHPO_4$, 70.36; NaCl, 20.82; KCl, 7.12;

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$MnSO_4 \cdot H_2O$, 0.641; $FeSO_4 \cdot 7H_2O$, 0.484; $MgCO_3$, 0.371; $ZnCO_3$, 0.120; $CuSO_4 \cdot 5H_2O$, 0.082; and KI, 0.0008. The same basic formula was used in experiments 2 and 3, with modifications in the supply of Mg, K and Na to give the desired variations.

Cation analyses were made by atomic absorption methods employing a Perkin Elmer Model 303 instrument. Phosphorus analyses were made by the AOAC colorimetric procedure.[1] The data were treated by analysis of variance procedures and all effects cited are significant at the 1% level.

In experiment 1, a test of effects of varying levels of Mg and K, 96 rats were used and assigned to 1 of 6 outcome groups based on vertical cage alignment. Within each outcome group rats were randomly assigned to treatment. Additions of $MgCO_3$ and of KCl were made at the expense of glucose, providing by analysis 4 levels of K (0.09, 0.27, 0.62 and 1.40% K) at each of 4 levels of Mg (40, 220, 650 and 1255 ppm Mg) except that through a mixing error the 0.27% K, 39 ppm Mg diet actually contained 0.62% K. All of the diets contained 0.50% Ca and 0.50% P.

In experiment 2, a test of varying levels of Mg and Na, 60 rats were randomly alloted to 12 groups of 5 animals each. The salt mixture used in the preceding experiment was altered by increasing its KCl and decreasing its NaCl content. Supplements of $MgCO_3$ and NaCl were made to provide the desired dietary variables. By analysis the diets contained 40, 215 and 1020 ppm Mg at 0.048, 0.497 and 0.927 % Na. All of the diets contained 0.27% K, 0.50% Ca and 0.51% Ca and 0.51% P.

In experiment 3, a test of excess Na and K as carbonates, a 2^3 factorial design was employed. A total of 40 rats was distributed randomly among the 8 diets. The basal diet contained 70 ppm of Mg, 0.3% K and 0.22% Na, the latter two being supplied as the chlorides in the mineral mixture. Additional levels of the carbonates were added to give 70 or 330 ppm of Mg and at each Mg level, 0.3% and 1.5% K and at each K level, 0.22% or 0.90% Na. The addition of K or Na as carbonates raised the cation-anion ratio of the diets from about 1.1:1 to 2.1:1, and the addition of both carbonates raised it further to 3.2:1.

RESULTS

Experiment 1. During the course of the experiment 13 rats died, 12 of them during the fourth week while being fed the lowest Mg diet (2, 4, 4 and 2 rats, respectively, on increasing levels of K). The thirteenth animal was fed the lowest level of K at 1255 ppm Mg, and died on the fifteenth day, having eaten and gained less well than the other 5 rats on the same treatment. It is judged that his atypical performance was not caused by the dietary treatment. In the Mg-deficient rats, erythema appeared on the fifth or sixth day and skin lesions on about the twelfth day in all animals except those on the lowest K level. These latter animals showed neither the erythema or severe skin lesions or convulsions typical of the other Mg-deficient groups. Potassium-deficient rats receiving supplemental levels of Mg showed no outstanding symptoms other than poor weight gain.

The quantitative data of this experiment are shown in tables 1 and 2. Gain and serum data are presented only for animals surviving to the end of the experiment. The remaining data represent means of 6 animals per treatment. Weight gain of the animals was depressed by deficiency of Mg and of K. A significant interaction between Mg and K reflects the fact that with the K-deficient diets each increase in Mg resulted in increased weight gain, (diets 1, 5, 9, 13), whereas in K-adequate diets increasing Mg above 220 ppm did not stimulate weight gain.

Serum Mg levels were normal only at the two highest Mg intakes and, within dietary Mg levels, were increased at the lowest level of K. Serum K was increased in dietary Mg deficiency and was significantly depressed in K deficiency except at the lowest level of dietary Mg. Serum P was decreased and serum Ca was increased by Mg deficiency but they were uninfluenced by dietary K level.

Muscle Mg was lowered in animals receiving the lowest Mg diet and was increased in K deficiency although this latter effect was small. Muscle K was depressed in K deficiency and in Mg deficiency of animals receiving adequate K

[1] Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.

TABLE 1

Weight gain and mineral composition of blood serum and muscle as affected by various levels of dietary magnesium and potassium

Diets			Daily gain	Serum				Muscle			
No.	K	Mg		Mg	Ca	K	P	Mg	Ca	K	Na
	%	ppm	g/day	mg/100 ml				mg/g dry wt			
1	0.10	40	1.42	0.60	14.0	33.5	10.3	1.06	1.56	13.4	8.1
2	0.62	40	1.86	0.48	12.5	41.6	9.1	0.99	0.44	14.0	8.4
3	0.62	40	1.89	0.47	12.8	35.5	9.6	1.00	0.52	12.9	7.6
4	1.40	40	1.82	0.51	11.4	38.1	9.4	1.00	0.50	14.0	8.2
5	0.10	220	2.93	1.90	10.8	23.5	11.1	1.26	0.33	12.6	6.7
6	0.27	220	5.96	1.51	11.3	32.3	12.0	1.19	0.30	18.1	4.4
7	0.62	220	6.07	1.45	11.0	35.7	11.5	1.19	0.27	18.5	4.2
8	1.40	220	5.42	1.35	11.4	35.2	12.4	1.19	0.27	18.3	4.0
9	0.10	650	3.32	2.68	10.8	23.1	11.3	1.23	0.35	12.3	6.7
10	0.27	650	5.74	2.08	11.0	32.6	11.4	1.19	0.28	17.3	4.5
11	0.62	650	5.85	2.11	11.4	33.1	12.2	1.19	0.27	17.5	4.6
12	1.40	650	5.78	1.97	11.2	34.1	11.2	1.19	0.27	18.4	4.0
13	0.10	1260	3.57	2.92	10.8	27.3	11.1	1.22	0.28	12.2	6.0
14	0.27	1260	5.03	2.26	10.9	34.2	12.3	1.22	0.28	18.0	4.1
15	0.62	1260	6.07	2.19	11.1	33.7	12.3	1.20	0.31	18.4	4.4
16	1.40	1260	5.14	2.15	10.7	33.2	11.9	1.19	0.27	17.8	4.1

TABLE 2

Mineral composition of heart, kidney and tibia as affected by various dietary levels of magnesium and potassium

Diets [1]	Heart				Kidney			Bone	
	Mg	Ca	K	Na	Ca	K	P	Mg	K
	mg/g dry wt				mg/g dry wt			% of ash	
1	0.73	2.28	13.0	9.4	13.03	12.7	23.1	0.24	0.12
2	0.67	3.81	12.1	8.6	4.94	13.5	18.5	0.22	0.24
3	0.70	0.94	12.8	9.3	1.45	12.8	16.4	0.24	0.24
4	0.74	3.05	12.4	9.7	0.89	13.3	17.2	0.21	0.20
5	0.88	0.38	12.4	9.0	0.44	11.2	16.1	0.81	0.39
6	0.83	0.28	12.6	6.9	0.80	12.8	15.8	0.62	0.45
7	0.80	0.30	12.5	6.6	0.50	13.0	15.2	0.62	0.55
8	0.84	0.33	13.2	7.0	0.58	13.3	16.1	0.59	0.40
9	0.85	0.36	11.7	8.6	0.44	11.5	16.5	1.00	0.44
10	0.83	0.32	12.7	6.4	0.44	13.1	15.8	0.81	0.57
11	0.84	0.32	13.0	7.0	0.44	13.5	16.1	0.80	0.53
12	0.82	0.31	12.8	7.2	0.41	13.2	15.6	0.77	0.57
13	0.84	0.42	11.5	9.3	0.49	11.1	15.4	1.08	0.47
14	0.84	0.31	12.7	7.5	0.44	13.3	16.0	0.90	0.60
15	0.84	0.31	13.2	7.0	0.43	13.4	15.7	0.86	0.57
16	0.86	0.31	13.4	6.8	0.45	13.2	15.6	0.85	0.58

[1] See table 1.

intake. Muscle Na was affected in the opposite direction to that of K, being increased in Mg deficiency and in K deficiency. Although the effects were not additive, Mg deficiency was accompanied by increased muscle Na more than was K deficiency. Muscle Ca was increased in Mg deficiency and was strongly increased by the combined deficiency of Mg and K.

The Mg concentration of the heart muscle was decreased in the Mg-deficient animals but was not different between the Mg-supplemented groups and was not affected by K intake. The heart Mg was in

all cases lower than the skeletal muscle Mg. Heart K was lower than skeletal muscle K of K-adequate animals and was not affected by dietary K or Mg. Na content of heart muscle was higher than that of skeletal muscle and was increased both in Mg deficiency and in K deficiency although the effects were not additive. Heart Ca was markedly increased in Mg deficiency, and among those animals receiving supplemental Mg, a small but significant increase in heart Ca accompanied K deficiency.

Kidney K was lowered in K deficiency except in Mg-deficient animals but was otherwise unaffected by Mg level of the diet. Kidney Ca was greatly increased by Mg deficiency but was not affected by the K level of the diet. The apparent great nephrocalcinosis of animals deficient in both Mg and K is largely the result of one kidney containing 54 mg Ca/g of dry matter.

Bone ash Mg was markedly decreased by Mg deficiency and was increased by K deficiency in those animals receiving supplemental Mg. Bone ash K was decreased both by Mg deficiency and by K deficiency.

Experiment 2. Animals on the lowest level of Mg all showed typical erythema in 5 to 6 days, irrespective of the Na level of diet, and one Mg-deficient animal at each Na level (except for 2 at the second level) died during the third week of the experiment. The quantitative data are shown in table 3. Weight gains were inhibited by Mg deficiency and by Na deficiency, there being no interaction between these minerals in this respect.

Each increasing level of dietary Mg increased serum Mg at all levels of dietary Na. A deficiency of dietary Na increased serum Mg at each Mg level. This effect was striking and entirely similar to that produced by a deficiency of K. Serum Ca was increased by Mg deficiency but was unaffected by dietary Na. Serum K was decreased in Mg-deficient animals receiving adequate Na, but in animals deficient in Mg and Na there was increased serum K. Serum Na was not affected by Na deficiency but was decreased by Mg deficiency.

The Mg in thigh muscle was decreased by Mg deficiency but unchanged in Na deficiency. Muscle K was lowered by Mg deficiency of animals receiving Na-adequate diets. Calcium in muscle was increased in Mg deficiency but was not affected by Na level of the diet. Muscle Na was increased by Mg deficiency.

Sodium levels did not affect mineral composition of the heart. As has been previously observed, Mg deficiency decreased heart Mg and increased Ca; it did not influence heart Na or K.

Sodium and also Mg deficiency decreased kidney Mg. Calcium in kidney was increased by Mg deficiency. Potassium in kidney was decreased in Mg deficiency, particularly at the lowest level of dietary Na.

Magnesium and K were increased in bone ash by Na deficiency. Mg deficiency markedly reduced bone ash Mg, and increased Ca in bone ash. Bone ash Na was decreased in Mg deficiency.

Experiment 3. In the third experiment the Mg content of the basal diet was increased somewhat above that in experiments 1 and 2 in order to prevent loss of animals due to Mg deficiency. This was successful in preventing mortality and yet permitted the production of a definite Mg deficiency as judged by the appearance of erythema in 7 to 8 days and skin lesions in 12 to 14 days, and in subnormal weight gains and serum Mg values which were improved in rats fed the higher Mg level. The quantitative data are shown in table 4.

The major effect on weight gain was brought about by the Mg level of the diet, although the effect of K addition was to decrease weight gain, especially of rats fed the higher levels of Mg. This was associated with a slightly rancid odor and relatively dark color of the feed containing K_2CO_3.

Serum Mg was decreased by dietary Mg deficiency and was also lowered by excess K, and by excess Na in the presence of a deficient Mg supply. Serum Ca was increased by the dietary Mg deficiency; Na and K were without effect on serum Ca. Serum K was decreased by Mg deficiency and increased by Na or K excess. Serum Na was elevated by dietary Mg deficiency.

Muscle Ca was increased by Mg deficiency. Muscle K was increased by K ex-

TABLE 3

Weight gain and tissue composition as affected by various levels of dietary magnesium and sodium

Diets	Na	Mg	Daily gain	Serum					Muscle				Heart			
				Mg	Ca	K	Na	P	Mg	Ca	K	Na	Mg	Ca	K	Na
	%	ppm	g/day	mg/100 ml					mg/g dry wt				mg/g dry wt			
1	0.048	40	2.18	0.82	11.9	29.6	305	9.0	1.08	0.36	19.9	4.1	0.87	0.72	13.9	7.8
2	0.497	40	2.89	0.59	11.0	22.2	304	8.4	0.88	0.48	15.2	6.5	0.80	0.64	13.0	7.2
3	0.927	40	2.43	0.59	10.9	26.1	303	8.8	0.96	0.49	16.8	6.8	0.92	0.88	13.6	6.8
4	1.22	40	2.78	0.59	11.0	25.0	306	9.1	0.93	0.33	17.5	6.8	0.79	0.75	12.4	7.2
5	0.048	215	4.28	2.18	10.1	25.9	314	10.1	1.21	0.23	21.3	2.8	0.98	0.24	13.2	6.3
6	0.497	215	5.96	1.52	10.5	26.2	314	9.9	1.20	0.25	20.4	3.0	0.95	0.22	12.5	6.5
7	0.927	215	6.07	1.34	11.1	26.9	320	11.2	1.23	0.24	18.9	3.3	0.95	0.24	12.6	6.0
8	1.22	215	5.71	1.28	11.0	28.5	326	11.1	1.22	0.22	18.8	3.4	0.93	0.25	12.6	6.5
9	0.048	1020	4.50	2.40	10.4	31.8	330	10.9	1.22	0.20	18.0	3.1	1.02	0.25	13.4	7.6
10	0.497	1020	5.71	2.07	10.3	27.6	332	12.1	1.23	0.22	18.4	3.1	1.00	0.25	13.0	7.6
11	0.927	1020	5.82	1.95	10.6	28.1	331	11.6	1.22	0.21	19.6	3.1	1.00	0.24	12.2	6.5
12	1.22	1020	5.93	1.97	10.3	29.1	329	11.0	1.18	0.21	19.6	3.3	0.97	0.25	12.1	7.3

Diets	Na	Mg	Daily gain	Kidney				Bone			
				Mg	Ca	K	Na	Mg	Ca	K	Na
	%	ppm	g/day	mg/g dry wt				% of ash			
1	0.048	40	2.18	0.73	3.54	11.4	7.1	0.25	38.3	0.26	0.91
2	0.497	40	2.89	0.76	3.21	12.2	7.6	0.26	38.2	0.22	0.88
3	0.927	40	2.43	0.77	1.26	12.6	9.7	0.22	38.3	0.10	0.84
4	1.22	40	2.78	0.76	2.89	13.0	10.4	0.25	37.9	0.08	0.86
5	0.048	215	4.28	0.77	0.32	12.3	7.1	0.76	35.8	0.37	0.87
6	0.497	215	5.96	0.86	0.97	12.9	7.2	0.60	36.2	0.25	0.94
7	0.927	215	6.07	0.84	0.82	13.3	7.1	0.55	36.4	0.24	0.95
8	1.22	215	5.71	0.83	1.20	13.1	7.6	0.54	37.2	0.26	0.97
9	0.048	1020	4.50	0.79	0.37	13.3	6.9	0.93	37.0	0.43	0.88
10	0.497	1020	5.71	0.90	0.36	13.5	6.7	0.86	37.5	0.33	0.92
11	0.927	1020	5.82	0.91	0.37	13.3	7.0	0.84	37.0	0.33	0.95
12	1.22	1020	5.93	0.96	0.37	13.4	7.1	0.83	36.8	0.33	0.94

TABLE 4

Weight gain and tissue mineral composition as affected by dietary magnesium, sodium and potassium carbonates

Diet	Mg	K	Na	Daily gain	Serum				Muscle			
					Mg	Ca	K	Na	Mg	Ca	K	Na
	ppm	*%*	*%*	*g/day*	*mg/100 ml*				*mg/g dry wt*			
1	70	0.30	0.22	4.25	0.80	10.7	24.7	322	1.23	0.27	17.8	4.16
2	70	1.50	0.22	4.18	0.67	11.0	27.9	319	1.22	0.29	18.9	3.95
3	70	0.30	0.90	4.33	0.65	10.9	29.0	323	1.15	0.28	19.3	4.35
4	70	1.50	0.90	3.92	0.59	10.9	29.7	317	1.16	0.30	19.5	4.28
5	330	0.30	0.22	6.18	1.87	10.3	28.4	292	1.20	0.26	18.4	2.93
6	330	1.50	0.11	5.85	1.62	10.0	30.8	300	1.26	0.24	19.3	3.15
7	330	0.30	0.90	6.40	1.80	10.2	30.3	294	1.21	0.24	18.0	3.24
8	330	1.50	0.90	5.51	1.59	10.3	31.0	298	1.25	0.24	18.9	3.27

Diet	Mg	K	Na	Daily gain	Kidney					Bone			
					Mg	Ca	K	Na	P	Mg	Ca	K	Na
	ppm	*%*	*%*	*g/day*	*mg/g dry wt*					*% of bone ash*			
1	70	0.30	0.22	4.25	1.06	6.21	13.8	8.7	19.5	0.25	38.16	0.15	1.00
2	70	1.50	0.22	4.18	1.09	19.50	13.6	8.5	24.5	0.24	38.16	0.19	1.01
3	70	0.30	0.90	4.33	1.02	15.41	14.4	9.0	22.8	0.24	38.64	0.15	1.01
4	70	1.50	0.90	3.92	1.12	10.24	14.6	8.5	21.3	0.27	38.82	0.06	0.94
5	330	0.30	0.22	6.18	1.17	0.47	13.7	6.9	16.3	0.81	37.14	0.29	0.99
6	330	1.50	0.11	5.85	0.96	0.44	14.2	7.0	16.8	0.74	37.08	0.25	0.97
7	330	0.30	0.90	6.40	0.97	0.46	13.7	6.7	16.7	0.75	36.70	0.28	1.00
8	330	1.50	0.90	5.51	1.01	0.57	13.9	7.0	17.3	0.68	36.74	0.29	0.98

cess, and by Na with the low Mg diet. Muscle Na was increased by Mg deficiency.

Magnesium composition of the kidney was not affected by treatment; a marked increase of Ca and P of the kidneys was evident in those animals fed the lower Mg diet, particularly those receiving supplemental Na or K carbonates. Kidney K was increased by excess Na only in rats fed the low Mg diet. Kidney Na was increased by Mg deficiency.

Magnesium of bone was decreased by Mg deficiency and was decreased by addition of K or Na to higher Mg diet. Bone Ca was increased by Mg deficiency and especially in the presence of excess Na. Bone K was decreased by Mg deficiency and was decreased in the bone of rats fed excess Na and K with low Mg diet. Bone Na was unaffected by treatment.

DISCUSSION

The results of these experiments are discussed, first, from the viewpoint of effects of deficiencies of the elements in question and, second, from the viewpoint of excess supply.

The effects of Mg deficiency on tissue mineral composition were, with few exceptions, consistent between the 3 experiments. In all tissues examined there was an increase in Ca and decrease in Mg except that in experiment 3 the Mg content of muscle and kidney was not affected, perhaps a result of the higher Mg content of the deficient diet. Serum Na was apparently decreased in experiment 2 and increased in experiment 3 as a result of Mg deficiency and serum K was increased in experiment 1 and decreased in experiment 2 and 3. No apparent cause of these discrepancies exists and a similar discrepancy between experiments was noted by Seta et al. (6). There was a consistent effect of Mg deficiency of decreasing muscle and bone K and increasing muscle Na. The increased Ca of Mg-deficient kidneys in experiment 3 was closely correlated with kidney P level ($r = 0.95$), hence indicating the lesion to result in formation of a calcium phosphate type of calculus.

The data of experiment 1 are in essential agreement with those of Whang and Welt (3) and of Manitius (4) and illus-

trate that certain of the changes in tissue mineral concentration caused by a Mg-deficient diet are similar to those induced by K deficiency. These include as a consequence of deficiency of either Mg or K, a decrease in muscle K and increase in muscle Na, a decrease in bone ash K, and increases of heart Na and Ca but no change in heart K.

Different effects of K and of Mg deficiency were also noted. Mg deficiency lowered blood serum Mg but K deficiency raised it; K deficiency did not affect Ca or P, whereas serum Ca was increased and P was decreased in Mg deficiency. Muscle Mg and Ca followed the same trends as did serum Mg and Ca although muscle Ca was increased by K deficiency in Mg-deficient animals. Potassium deficiency did not affect kidney Ca, whereas a marked increase of kidney Ca was a consequence of Mg deficiency. Bone ash Mg, like that of serum and muscle Mg, was increased by K deficiency but reduced by Mg deficiency.

It is obvious, then, that Mg deficiency does not completely replicate K deficiency in its effects on tissue mineral composition. The areas of similarity involve muscle K and Na, elements which are quantitatively more important in muscle than are Ca and Mg. Mg deficiency actually decreased muscle K to the same extent as did K deficiency and increased muscle Na more than did simple K deficiency. The major area of dissimilarity relates to soft tissue Ca in which the effects of Mg deficiency were much greater than those of K deficiency. It may thus be concluded that the Ca changes are a direct effect of Mg deficiency and that the increases in Na are secondary to the deficiency of K produced either directly or due to an Mg deficiency and represent a homeostatic reaction whose purpose is to maintain normal osmolar concentrations within the muscle cells.

The combined deficiency of Mg and K prevented the appearance of acute Mg deficiency symptoms, perhaps as a result of the very small feed intake and gain; although the combined deficiency of Mg and Na permitted only slightly better gains, these animals did exhibit the typical erythema and eventual skin lesions.

These data indicate that in general the first level of supplementation with either Mg or K prevents the tissue changes engendered by the deficient diets. The apparent exceptions to the above statement relate to the fact that the first supplementation of Mg raised total Mg to 220 ppm, which provided maximal weight gain but not maximal Mg concentration in serum or in tibia. This is in agreement with our earlier observations (7) that approximately 500 ppm Mg are required to maximize these criteria of Mg nutriture. It is apparent, also that increasing the level of Mg or of K severalfold above the requirement did not allay the deficiency of the other mineral except that increase of K intake by Mg-deficient animals appeared to decrease kidney calcification.

There were few areas of similarity in change of tissue minerals accompanying Na or Mg deficiency. Both Na and Mg deficiencies lowered kidney Mg and K, and bone ash Na. In several other respects the effects were of opposite direction. For example, Na deficiency increased serum Mg (as did K deficiency), increased muscle K (with Mg-deficient diet) and bone Mg (with Mg-adequate diet) and decreased kidney Na. Mg deficiency, on the other hand, had generally opposite effects in these situations.

As in the case of supplemental K, there was little effect of Na supplement beyond that required to overcome Na deficiency as judged by weight gain. With respect to these levels, a deficiency of Na, like that of K, increased serum Mg, decreased kidney K and increased bone Mg of animals receiving adequate Mg. Tissue constituents affected in opposite directions by K and by Na deficiency included an increase in serum K, and in muscle K of Mg-deficient animals as a result of Na deficiency.

Excesses of Na or of K did not affect mineral composition of the heart, of the Mg-normal kidney or of Mg-deficient bone. In Mg-normal bone, excess Na or K as carbonates, but not as chlorides, decreased Mg; Na as the carbonate, but not K, decreased Ca in Mg normal bone. In the Mg-deficient kidney, Ca was increased by excess K or Na as carbonates, but not as chlorides.

In Mg-normal muscle the only effect of excess mineral ions was an increase in K when supplied as K_2CO_3. In the Mg-deficient muscle excesses of K and of Na as carbonates increased K. Excess K and Na as carbonates decreased serum Mg in Mg deficiency, whereas K as carbonate decreased serum Mg in the presence of adequate dietary Mg.

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Movement of Physiological Levels of Selenium from Soils through Plants to Animals [1,2]

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ABSTRACT This study was undertaken to trace quantitatively the movement of low levels of Se in the soil-plant-animal cycle and to determine the effect of Se moved through this cycle upon the incidence of white muscle disease (WMD). Se, added as Na_2SeO_3, to a low-Se soil was taken up by alfalfa. When alfalfa grown on a Se-treated soil was fed to sheep, protection from WMD was evidenced, under conditions where WMD was prevalent in lambs from ewes fed alfalfa grown on comparable untreated soil. Se from the alfalfa was retained in the tissues of lambs, and a concentration of 0.21 ppm Se in the livers of lambs is evidently near to a minimal required level for this element. Ewes fed alfalfa containing 2.6 ppm Se for 150 days, and then fed a low Se diet, transmitted protective levels of Se to lambs born 10 months later. Residual effects of Se in the soil upon the Se content of alfalfa were evident 2 years after Se application. The use of soil applications of Se to protect animals from WMD is inefficient in terms of the amount of Se required and involves some hazard from Se toxicity.

The occurrence of selenium toxicity in a number of species has been shown to result from the movement of Se from highly seleniferous soils through plants to animals (1, 2). At the other end of the physiological scale, there is a growing body of evidence that minute amounts of Se in feeds are necessary for prevention of degenerative processes, including white muscle disease (WMD) in ruminants (3, 4). Quantitative information on the movement of the low amounts of Se essential for the prevention of WMD is, however, generally lacking.

The objective of this study was to trace quantitatively the movement of low levels of Se in the soil-plant-animal cycle and to determine the effect of Se moved through this cycle upon the incidence of WMD. The procedure followed was to measure the concentrations of Se in alfalfa grown on soils to which different levels of Se had been added. This was studied first under greenhouse conditions and later in the field. Ewes were fed alfalfa grown on untreated and on Se-treated soil. The incidence of WMD and tissue Se concentrations in lambs born to these ewes were determined. Residual effects of Se in the soil and in the animals were also measured.

EXPERIMENTS AND RESULTS

Site of the experiment. A ranch in Jefferson County, Oregon, was selected as the field site for alfalfa production. Jefferson County is an area where WMD is enzootic, and the feeding of alfalfa from this ranch had resulted in WMD in earlier experiments (5).

The soil on the field site is Madras sandy loam, a Mollic Durargid (Brown) soil (6) formed from mixed volcanic materials. This ranch has been irrigated since 1949 and has received heavy applications of phosphorus and sulfur while being cropped to alfalfa, potatoes, and grain.

Uptake of Se by alfalfa in the greenhouse. A large bulk sample of the surface soil from the field site in Jefferson County, Oregon, was screened and mixed with 250 ppm of superphosphate and placed in polyethylene pots (2.72 kg air-dried soil per pot). Rooted cuttings of alfalfa were established in these pots and grown in the greenhouse with supplemental fluorescent light until the first

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bloom stage. At this stage, the tops of the plants were cut off and discarded, and Se at varying rates was added to the pots of soil. Three replicate pots of each rate of Se addition were used. The Se was added by injecting water solutions of Na_2SeO_3 labeled with ^{75}Se into the potted soil about 5 to 7 cm below the surface. The regrowth of the plants was cut 5 weeks after the Se additions were made (one-third bloom stage), and the uptake of Se was determined by counting the ^{75}Se in the air-dried and ground plant material, in comparison with similar counts of the original tagged Na_2SeO_3 solution. This procedure disregards any contribution of the native Se in the soil to the Se content of the plant. The plants were then allowed to regrow for an additional 6 weeks, and measurement of Se uptake was repeated.

Concentrations of Se in the alfalfa, resulting from different rates of addition of Se to the soil, are shown in table 1. Various studies have shown that 5 to 15 ppm is the minimal concentration of Se in animal diets necessary to depress growth or cause chronic toxicity (2). Rates of Se addition of 1.5 ppm and above have, therefore, resulted in potentially detrimental concentrations of Se in the alfalfa. At the higher rates of Se addition, a decline in Se content of the second cutting of the alfalfa was noted. No effects of the Se treatments upon the growth of the alfalfa were noted.

Uptake of Se by alfalfa under field conditions. The next experiment consisted of the measurement of the uptake by alfalfa of Se added to the soil under field conditions on the central Oregon experimental site. Three rates of Se application, zero, 0.15, and 0.5 ppm were used. (These Se additions were, respectively, 336 and 1120 g/hectare, on the assumption that the surface tilled layer, 18 cm deep, of the field soil would weigh about 2,240,000 kg/hectare.) The field used for this study had a 2-year-old stand of alfalfa on it.

TABLE 1

Concentration of Se in alfalfa as affected by the rate of Se added as labeled Na_2SeO_3 to Madras sandy loam in the greenhouse

Treatment no.	Se added to soil	Se in alfalfa tops [1]	
		First cutting	Second cutting
	ppm	*ppm*	*ppm*
1	0	—	—
2	0.015	0.09	0.06
3	0.050	0.26	0.21
4	0.150	0.94	0.99
5	0.50	3.14	2.64
6	1.50	7.94	5.56
7	5.00	28.6	14.4
8	15.00	75.9	42.0

[1] Calculated from ^{75}Se in the plant, disregarding uptake of any native Se in the soil.

The Se applications to the field soil were made by injecting a water solution of Na_2SeO_3 into the soil at a depth of about 10 cm with a liquid fertilizer applicator. The injectors were spaced 35 cm apart. The plots receiving the different Se rates were arranged so as to prevent movement of Se from one plot to another in irrigation or drainage water. This arrangement eliminated the possibility of cross contamination of the plots through movements of Se in the irrigation water, but it also meant that the different rates of Se application could not be randomized and replicated in the same field. As a further safeguard, individual plots were made 0.8 hectare in area to permit wide, unused borders.

Application of Se to this field was made in April, 1962, before the new growth of alfalfa had started. Samples of the top growth of the alfalfa were taken from each plot immediately before the harvest of each cutting in 1962 and 1963. The samples represented 20 locations from the central part of each plot. These samples were then dried at 50° and analyzed for Se, using a fluorometric method (7).

The effects of rates of Se added to the field soil upon the Se content of successive cuttings of the alfalfa over a 2-year period are shown in table 2. The Se concentrations in the alfalfa were somewhat lower than expected on the basis of the greenhouse experiment, but even so, measurable uptake of the Se added to the field soil was evident. The addition of 0.5 ppm Se to the soil resulted in a 1962 crop of alfalfa containing Se concentrations higher than the 0.05 to 0.10 ppm observed previously in forages that caused WMD in sheep (4, 5, 8–10). By 1963, the Se content of the alfalfa had declined to near-marginal levels, even from the highest rate of Se applied in 1962. The Se content of the 1962 third cutting of alfalfa from the untreated plot

TABLE 2

Effect of Se added to the soil upon the Se content of alfalfa grown under field conditions

	Se added to soil in April 1962,[1] ppm		
	0	0.15	0.50
	Se content of alfalfa		
	ppm	*ppm*	*ppm*
First cutting, 1962	0.04	0.10	0.29
Second cutting, 1962	0.05	0.10	0.34
Third cutting, 1962	0.09	0.13	0.25
First cutting, 1963	0.01	0.07	0.12
Second cutting, 1963	0.02	0.06	0.13

[1] Se injected into the soil as a water solution of Na_2SeO_3.

was higher than normally present in central Oregon. This may be due to the fact that the 1962 third cutting was taken at a very young stage of growth.

Production of selenized alfalfa for feeding experiments. The next step was the production of sufficient alfalfa, from Se-treated and untreated soils, for feeding trials to test the effect of Se taken up from the soil by alfalfa upon the incidence of WMD in sheep. For this step, a second field on the Jefferson County, Oregon, ranch was treated with Se in 1963. This second field consisted of a one-year-old stand of alfalfa growing on the same soil type that occurred on the field treated with Se in the 1962 experiment. A 2-hectare plot in this second field was treated with Se at the rate of 1 ppm Se (2240 g/hectare), and a comparable plot in this field was left with no Se treatment. The 1-ppm Se rate was selected in an attempt to produce alfalfa with about twice the Se content of that produced when 0.5 ppm Se was applied in 1962, and to reach a level of available Se in the soil that might have a residual effect in succeeding years.

In 1963, Se was applied to the field in late June, *after* the first cutting of alfalfa was removed and just prior to irrigation of the second cutting. The method of applying Se to the soil was the same as used in 1962, and the Se-treated plot was again located so that no cross-contamination of the untreated plot could occur through the movement of Se in irrigation or drainage waters. The second cutting of alfalfa from the treated and untreated plots was harvested, field-dried, and baled in August, 1963, six weeks after the Se application was made. Harvesting was conducted so that all machinery moved from the untreated plot to the treated plot, and each bale of alfalfa was labeled before being removed from the plot where it was grown. The alfalfa was then moved to Corvallis, Oregon, where it was chopped into 10-cm lengths and stored in separate lofts for use in a feeding experiment conducted during the winter of 1963–64.

The stored alfalfa was sampled at 2 different times during the winter of 1963–64. Each sample consisted of 2 kg of chopped alfalfa removed from different parts of each storage loft. These 2-kg samples were dried at 50°, ground and subsampled for Se analysis (7).

The Se content of the 2 samples of the alfalfa grown on the untreated plot was 0.01 and 0.04 ppm. The Se concentrations in the 2 samples of alfalfa grown on the plot treated with 1 ppm Se in June 1963 were 2.7 and 2.6 ppm Se. These Se analyses were in good agreement with results of nondestructive neutron activation analysis of the same materials. Seventy-seven per cent of the Se in the alfalfa from the Se-treated plot was insoluble in cold 5% trichloroacetic acid, and was probably present as seleno-amino acids in proteins.

The Se concentration in the alfalfa from the plot treated with 1 ppm Se in June 1963 was higher than expected on the basis of the results from the rates of Se application used in 1962. This difference appears to be due to the fact that in 1963 the Se was applied in the summer when the alfalfa was growing rapidly, whereas the 1962 treatments were applied in early spring when the alfalfa was nearly dormant, and there was more time for the added Se to revert to insoluble forms in the soil prior to the period of rapid growth of alfalfa.

The 1963–64 feeding experiment. The second-cutting alfalfa, produced on the Se-treated and untreated plots (1963 treatments), was fed to ewes during the winter of 1963–64, and the effect of the Se taken up from the soil by the alfalfa upon the incidence of WMD in their lambs was determined. Mature Corriedale ewes for this experiment were obtained from Wallowa County, Oregon (a low-Se area), and placed on pasture at Corvallis, Oregon in August,

1963. Samples of the grass and clover from this pasture contained less than 0.01 ppm Se. The ewes were maintained in one group and bred while on this pasture during the period September 15 — November 1, 1963.

On November 15, 1963, the ewes were allotted at random to 4 pens of 10 ewes each. Two of these pens were fed the second-cutting alfalfa from the plot treated with 1 ppm Se in June 1963, and two were fed the alfalfa from the comparable untreated plot. Pens of ewes fed alfalfa from the untreated plot were separated from those fed alfalfa from the treated plot by a vacant pen in order to minimize cross-contamination of the feed. The alfalfa was fed at the rate of 1.8 kg/ewe/day, and distilled water and Se-free salt were offered for ad libitum consumption. From December 15 to the end of the experiment (May, 1964), the ewes received 114 g of oats/head daily. These oats contained 0.03 ppm Se.

The lambs were born in February and March, 1964, and any lambs that died before reaching 6 weeks of age were necropsied for evidence of WMD. At 6 weeks of age, all lambs were weighed and serum glutamic oxaloacetic transaminase (SGOT) levels were determined.[5] Skeletal and heart muscles of each lamb were examined for visible evidence of WMD. Sections of the skeletal (semitendonosus) muscle and heart muscle were examined for calcification and other microscopic lesions of WMD (11). The kidney, liver, and semitendonosus muscle of randomly selected lambs from each treatment were lyophilized and their Se concentrations determined (7).

The results of the 1963–64 feeding experiment (table 3) show that Se added to the soil and taken up by alfalfa protected lambs from WMD and elevated SGOT levels, whereas a high incidence of WMD and elevated SGOT levels was observed in lambs from ewes fed alfalfa from the untreated plot.

The data from the 2 separate lots of ewes fed each type of hay have been added

[5] Sigma Technical Bulletin 1957 A simplified method for the clinical determination of serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase at approximately 500 mμ. Sigma Chemical Company, St. Louis.

TABLE 3

Effect of Se taken up from the soil by alfalfa upon the incidence of white muscle disease (WMD), elevated SGOT levels, and tissue Se concentrations in lambs from ewes fed alfalfa grown on Se-treated and untreated soil (1963–64 exp.)

Se applied to soil in June 1963, ppm	0	1
Se in alfalfa cut in August 1963, ppm dry basis	0.01—0.04	2.6 —2.7
No. of ewes fed	20	20
No. of lambs born	29	32
No. of lambs dead prior to 6 weeks of age:		
With WMD lesions	7	0
Without WMD lesions	3	6
No. of lambs surviving to 6 weeks of age:		
With WMD lesions	13	0
Without WMD lesions	6	26
No. of 6-week-old lambs with elevated SGOT [1]	11	0
Wt of 6-week-old lambs, kg	12.3 ±2.5 [2]	11.8 ±3.4 [3]
Se in tissues of 6-week-old lambs:		
Skeletal muscle, ppm dry basis	0.04 ± 0.01 [4]	2.43 ± 0.21 [5]
Liver, ppm dry basis	0.10 ± 0.03 [4]	14.7 ±2.4 [5]
Kidney, ppm dry basis	0.83 ± 0.14 [4]	7.7 ±2.0 [5]

[1] Elevated level of SGOT = value above 200 Sigma Frankel Units.
[2] Mean ± SD for 18 lambs.
[3] Mean ± SD for 25 lambs.
[4] Mean ± SD for 12 lambs, of which 6 had WMD lesions and 6 had none.
[5] Mean ± SD for 5 lambs.

together for table 3. The weights of the lambs raised by the ewes fed the 2 different types of alfalfa were essentially the same. The Se concentrations in the liver and kidney of lambs from ewes fed alfalfa from the untreated plot were lower than those reported by Cousins and Cairney (12) for lambs affected with delayed WMD, and also lower than the Se levels found in Se-responsive lambs by Andrews et al. (9).

Accumulation of Se in the tissues, especially the liver and kidney is evident in lambs from ewes fed the high-Se alfalfa, but the levels of Se observed in the tissues of these lambs were lower than those reported in cases of Se toxicity. Glenn et al. (13) reported levels up to 27 ppm Se (wet basis) in the livers of ewes suffering from "selenate toxicosis."

The Se concentration was higher in the kidney than in the liver of lambs from ewes fed the alfalfa from the plot with no Se treatment. This situation was reversed in the lambs from the ewes fed alfalfa from the Se-treated plot.

In table 3, the 12 lambs selected for tissue Se analysis from the ewes fed alfalfa from the untreated plot included 6 lambs with no WMD lesions and low SGOT levels and 6 lambs with WMD and high SGOT levels. The Se content of the tissues from these 2 types of lambs was essentially the same.

The 1964–65 feeding experiment. No additional application of Se was made to the field in 1964, but the second cutting of alfalfa from this field was harvested in August, 1964, and fed to ewes during the winter of 1964–65 to measure the residual or carryover effects of the 1 ppm Se added to the field in 1963. The 1964 second cutting of alfalfa from the plot treated with 1 ppm Se in 1963 contained 0.43 and 0.57 ppm Se on the basis of 2 samples of it drawn from the storage lofts at 2 times during the winter of 1964–65. These values represent a sharp decline from those observed in the 1963 second cutting from the same plot, but they are still above the 0.05 to 0.10 ppm considered to be necessary for prevention of WMD (4, 5, 8–10). The 1964 second cutting of alfalfa from the untreated plot contained less than 0.01 ppm Se.

The 1964–65 feeding trial was conducted by essentially the same procedure as used in 1963–64. A new group of ewes from the same Wallowa County ranch that provided the 1963–64 ewes was obtained for the 1964–65 feeding experiment. From August, 1964, through the end of the breeding season on November 1, 1964, they were grazed on the same low-Se pasture at Corvallis. The diet of the ewes during the gestation period and until the lambs were 6 weeks old was again 1.8 kg of alfalfa and 114 g of oats/head daily. The oats were from the same lot as those used in the 1963–64 experiment, and the ewes were again watered with distilled water and given access to Se-free salt.

The results (table 4) show that Se taken up by alfalfa in 1964 from the 1 ppm added to the soil one year previously protected the lambs from WMD and the development of high SGOT levels. The concentrations of Se in the tissues of the lambs from ewes fed the alfalfa from the Se-treated plot were lower than those from ewes fed the high-Se alfalfa in 1963–64. The Se concentration in the liver was higher than that in the kidney of the lambs from the ewes fed alfalfa from the Se-treated plot, but as in the 1963-64 experiment the reverse was true in the lambs from the ewes fed alfalfa from the untreated plot. Thus, the Se concentration of the liver is a more sensitive indicator of the Se level of the ewe's diet than is the Se content of the kidney. This is in agreement with the observations of Andrews et al. (9).

Residual effects of Se in the ewes. Twelve ewes fed alfalfa (2.6–2.7 ppm Se) produced on the Se-treated plot in 1963 were held over after the 1963–64 feeding experiment. These 12 ewes were kept on the low-Se (less than 0.01 ppm) pasture at Corvallis during the summer of 1964, and were bred while on this pasture in September and October of 1964. They were then fed on the 1964 second cutting alfalfa produced on the untreated plot during gestation and the early post-partum period. They also received the low-Se oats and salt. Thus, their diet from August of 1964 until their lambs were 6 weeks old in April, 1965, was identical with that of the new ewes fed alfalfa from the untreated

TABLE 4

Effect of Se added to the soil in 1963 upon the Se content of the 1964 second cutting of alfalfa and upon the lambs from ewes fed 1964 crop alfalfa grown on Se-treated and untreated soil (1964–65 exp.)

Se applied to soil June 1963, ppm	0	1
Se in alfalfa cut August 1964, ppm dry basis	<0.01	0.43—0.57
No. of ewes fed	20	20
No. of lambs born	25	21
No. of lambs dead prior to 6 weeks of age:		
With WMD lesions	4	0
Without WMD lesions	0	1
No. of lambs surviving to 6 weeks of age:		
With WMD lesions	10	0
Without WMD lesions	11	20
No. of lambs with elevated SGOT [1]:		
At 2 weeks of age	5	1
At 6 weeks of age	8	0
Wt of 6-week-old lambs, kg	14.9 ±3.1 [2]	14.7 ±3.4 [3]
Se in tissues of 6-week-old lambs [4]:		
Skeletal muscle, ppm dry basis	0.02±0.02	0.85±0.16
Liver, ppm dry basis	0.04±0.02	4.02±0.90
Kidney, ppm dry basis	0.52±0.15	3.28±1.04
Heart, ppm dry basis	0.03±0.02	1.53±0.15

[1] Elevated SGOT = value in excess of 200 Sigma Frankel Units.
[2] Mean ± SD for 16 lambs.
[3] Mean ± SD for 15 lambs.
[4] Mean ± SD for 6 randomly selected lambs from each treatment.

plot in the 1964–65 feeding experiment and shown in the left-hand column of table 4.

These 12 ewes carried over from the 1963–64 experiment gave birth to 16 lambs in the spring of 1965. In 13 of these lambs that survived to 6 weeks of age, there was only one case of WMD, and it was not evident until the muscle was examined microscopically. This degree of protection from WMD is in sharp contrast with the high incidence of WMD in lambs from the new ewes brought in from Wallowa County, Oregon, in the summer of 1964 and fed the same diet during gestation and early growth of the lambs. The livers of the 1965 lambs, from the ewes fed alfalfa containing 2.6 to 2.7 ppm Se in the 1963–64 experiment and carried over on low-Se diets in 1964-65, contained 0.21 ± 0.04 ppm Se, and the skeletal muscle contained 0.05 ± 0.01 ppm Se (average ± SD for 6 randomly selected lambs). It is evident, then, that Se accumulated by the ewes during the 1963–64 feeding experiment was transferred to their lambs one year later in amounts sufficient to provide substantial protection from WMD.

The concentration of 0.21 ppm Se in the liver of the lambs from the ewes fed high-Se alfalfa in 1963–64 and low-Se alfalfa in 1964–65 appears to be near the critical minimal level for 6-week-old lambs, since these lambs were essentially free of WMD, whereas a high incidence of WMD was observed in the groups of lambs with lower levels of Se in the liver. This critical level is in very close agreement with data of Cousins and Cairney (12) who reported 0.18 ppm Se in the liver of WMD lambs and 0.28 ppm Se in the liver of normal lambs.

The carryover of Se in the ewes, and its transfer in protective amounts to the lambs one year after the ewes were fed alfalfa containing 2.6 to 2.7 ppm Se, suggests that animals must be maintained with low-Se diets for a considerable period

of time in order to pre-condition them for use in experiments designed to evaluate Se supplementation techniques.

Residual effects of Se in the soil. The alfalfa growing on the plot treated with 1 ppm Se in 1963 was sampled in June, 1965, just prior to harvest of the first cutting, and again in August, 1965, just prior to the second cutting. The first-cutting sample contained 0.39 ppm Se, and the second-cutting sample contained 0.36 ppm Se. Thus, the Se content of the alfalfa grown on the Se-treated plot was still above recognized minimal protective levels in animal diets 2 years after the application of 1 ppm Se. Furthermore, since the Se concentration in the alfalfa in 1965 was only slightly less than in 1964, it may be that this Se concentration 2 years after treatment represents a near equilibrium level of plant uptake from stable, slowly soluble forms of Se that have developed in the soil since the Se was applied. If this is true, the Se treatment of the soil may be effective in maintaining the Se concentration in the alfalfa above minimal critical levels for an additional period of years.

DISCUSSION

Under the conditions of the experiments reported here, the alfalfa produced on Se-treated plots should be very similar to that produced on the untreated plots in all respects except Se concentration. The high incidence of WMD in the lambs from ewes fed alfalfa grown on the untreated plot can, therefore, be ascribed to a deficiency of available Se in the soil. This deficieny can be corrected by application of soluble Se to the soil.

The levels of vitamin E, inhibitors to Se or vitamin E (14, 15), sulfur (16, 17), and any other constituents except Se that might affect the incidence of WMD should be the same in the diets of ewes fed alfalfa from the Se-treated plot as from the untreated plot. (In the case of sulfur, this similarity was confirmed by analyses of the 2 types of alfalfa. Sulfur concentrations in various samples of the alfalfa fed to ewes ranged from 0.26 to 0.29% S.)

The application of Se to the soil is, however, an inefficient method, in terms of the amount of Se required, of meeting the Se requirements of animals. Much of this inefficiency is due to the low percentage of the applied Se that is taken up by the plants. The amounts of Se taken up from the Se-treated soil by the harvested alfalfa in the experiments described here can be calculated on the basis of the yields and Se concentrations of the 5 cuttings of alfalfa harvested to date from the plot treated with 1 ppm Se in June, 1963. On the basis of these calculations, it is estimated that less than 2% of the Se applied in June, 1963, had been taken up from the soil by the 5 cuttings of alfalfa harvested since the Se was applied. The uptake of the applied Se may, however, continue in future years.

The 5 cuttings of alfalfa harvested since 1 ppm, or 2240 g, Se/hectare were applied in June, 1963, have yielded a total of 29,146 kg of alfalfa/hectare. At the rate of feeding used in these experiments (1.8 kg/ewe/day for 150 days), this amount of alfalfa would feed 108 ewes and protect the 124 lambs expected from them from WMD, assuming that the 1965 crop (0.39 and 0.36 ppm Se) has the same protective effect as the 1963 and 1964 harvests. Since the alfalfa produced on the plot treated with 1 ppm Se has contained substantially more than the critical minimal levels of Se, the protective effects against WMD of the total yield of 1 hectare might be extended to more lambs, possibly to 500 in all, by blending the alfalfa from the Se-treated plot with low-Se feeds. But, studies of the use of Se injections indicate that 1 or 2 mg/lamb used as individual injections would provide the same degree of protection from WMD (18). The use of Se injections is, therefore, much more efficient in terms of the amount of Se used than is the application of Se to the soil to protect lambs from WMD. It is to be recognized that long-term residual effects of the Se in the soil may increase the efficiency of soil application of Se, but since Se is a rare and costly element, methods of protecting animals from Se-responsive diseases that are efficient in terms of the amount of Se required over short periods of time may be preferred.

The use of soil applications of Se to control Se-responsive diseases of livestock poses some hazard due to the possibility that crops grown on Se-treated soils may

contain toxic levels of Se. Grant (19) observed interference with conception in ewes grazed on pastures sprayed with Na_2SeO_3 at the rate of 2 oz. Se/acre (140 g/hectare). In Grant's work high levels of Se in the forage may have been due to Se adhering to the leaves and stems of the forage. The variability of Se uptake under different conditions from the same soil, as evidenced by comparison of greenhouse and field data, dictates a need for rigid control procedures and precautions against overuse in any practical consideration of soil applications of Se. A form of Se less soluble than Na_2SeO_3 might, however, have a wider margin of safety for use as a soil application.

Finally, it should be emphasized that these experiments were conducted on just one kind of soil, and there are many different kinds of soil in the Se-deficient areas of the United States. Some of these soils may react with Se in a different manner from the soil used in these experiments.

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Comparative Production of Carious Lesions in the Occlusal Sulci and on the Smooth Surfaces of the Molars of Caries-susceptible Rats [1,2]

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ABSTRACT Semi-purified diet 2700 caused a relatively infrequent production of carious lesions on the smooth surfaces of the molars of Harvard and mutant albino rats which are susceptible to high incidences of carious lesions in the occlusal sulci. The use of several diets composed mainly of white bread and whole wheat flour resulted in substantial increases in the incidence of smooth surface lesions. In some cases these diets simultaneously caused significant decrease in the incidence of lesions in the occlusal sulci, while in other situations the incidence of both types of lesions was simultaneously high. The rats of the mutant albino strain tend to be more prone to develop the carious lesions on the smooth surfaces than rats of the Harvard strain. In addition, the distribution and characteristics of the smooth surface lesions tended to differ between the 2 strains, although the responses to the various diets were closely parallel.

In our dental caries studies over the past 20 years, we have been concerned primarily with the etiology of the carious lesions which occur in the occlusal sulci of rat molars as a result of the feeding of purified diets, such as our diet 2700 and various modifications (1–3). Our strains of caries-susceptible rats almost invariably did not develop lesions on the smooth surfaces of the molars even when widely varied diets were used. In the 1950's when McClure and co-workers (4–6) were studying the ability of various skim milk-cereal diets to produce carious lesions on the smooth surfaces, the same diets under our experimental circumstances were incapable of producing comparable lesions even though sulcal lesions progressed rapidly.[3] More recently a series of borderline protein deficiencies, produced by modifications of diet 2700, resulted in a low and somewhat sporadic incidence of smooth surface lesions in our caries-susceptible strains (7). Therefore this welcome but unexpected observation permitted us to make a re-examination of the ability of skim milk-cereal diets to produce smooth surface lesions in our Harvard and mutant albino caries-susceptible strains. The basic consideration in undertaking these studies was simply to attempt to define whether there were different nutritional and dietary aspects of the etiology of sulcal and smooth surface lesions under our experimental conditions. One of these conditions was that we would not deliberately introduce microorganisms which were not ordinarily present in the oral flora of our strains.

EXPERIMENTAL METHOD

The first experiment was conducted with rats of the Harvard caries-susceptible strain and consisted of 5 groups with littermate distribution among groups. The rats in the first group served as controls and were maintained with our cariogenic diet 2700 (8). The second through fourth groups were maintained with diets 390V, 390C, 374V and 374C, respectively. The composition of these diets is shown in table 1. Diets 390V and 390C were modifications of the whole wheat flour diet 291 of McClure and Muller (6), while diets 374V and 374C were modifications of their white bread diet 286. In our diets the symbol V indicated a supplement of B-complex vitamins comparable to the level in our diet 2700, as we did not wish to complicate interpretation of the results by reason of

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[3] Unpublished experiments, J. H. Shaw.

TABLE 1

Composition of diets (in grams)

	2700	374V	374C	390V	390C	391V	490V	590V	591V
Sucrose	670	—	—	—	—	—	—	—	—
White bread	—	600	720	—	—	—	—	—	—
Vitaminized white bread [1]	—	200	—	—	—	—	—	—	—
Whole wheat flour	—	—	—	600	720	420	580	420	240
Vitaminized whole wheat flour	—	—	—	200	—	200	200	200	200
Glucose monohydrate [2]	—	180	180	180	180	360	180	180	360
Vitaminized casein [3]	80	—	80	—	80	—	—	—	—
Casein	160	—	—	—	—	—	—	—	—
Skim milk powder	—	—	—	—	—	—	—	180	180
Calcium carbonate	—	13.7	13.7	12.5	12.5	12.5	12.5	12.5	12.5
Sodium chloride	—	5	5	—	—	—	—	—	—
L-Lysine·HCl	—	10	—	10	—	10	30	10	10
Salt mixture [3]	40	—	—	—	—	—	—	—	—
Vitaminized corn oil [3]	50	—	—	—	—	—	—	—	—
Whole liver powder	40	—	—	—	—	—	—	—	—
Cellulose [4]	150	—	—	—	—	—	—	—	—
Total ingredients	1190	1008.7	998.7	1002.5	992.5	1002.5	1002.5	1002.5	1002.5

[1] B-complex vitamins added in same amounts to 200 g of bread or flour as were contained in the 80 g of vitaminized casein used as the B-complex vitamin carrier in diet 2700.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] J. Dent. Res., 26: 47, 1947.
[4] Cellu Flour, The Chicago Dietetic Supply House, Inc., Chicago.

B-complex deficiencies. The symbol *C* indicated an 8% supplement of crude casein to overcome at least partially the low protein concentration and the low biological value of the protein in these diets. The casein supplement was made at the expense of either whole wheat flour in diet 390C or white bread in diet 374C. The latter 4 diets were supplemented by a concentrate of vitamins A and D that was given orally twice weekly at levels to provide adequate nutriture. The rats were placed on experiment at 21 days of age and killed after an additional 70 days on experiment.

The second experiment was identical to the first except that caries-susceptible rats of the mutant albino strain were used.

The third experiment was conducted with rats of the Harvard caries-susceptible strain and consisted of 6 groups with littermate distribution among groups. The rats in the first group served as controls and were fed diet 2700. The rats in the second group were fed diet 390V as a repetition of the second group in the first experiment. Groups 3 through 5 were fed diets 391V, 490V, 590V and 591V, respectively. The composition of these diets is shown in table 1. In diet 391V, the glucose monohydrate [4] content had been increased from 18 to 36% at the expense of whole wheat flour. In diet 490V, the lysine concentration was increased from 1.0 to 3.0% at the expense of whole wheat flour. In diet 590V, 18% of skim milk powder was introduced at the expense of whole wheat flour. In diet 591V, the glucose monohydrate concentration was increased to 36% and 18% of skim milk powder was introduced, both at the expense of whole wheat flour. Again each rat was given twice weekly an oral supplement with adequate amounts of vitamins A and D. The rats were placed on experiment at 21 days of age and killed after an additional 60 days on experiment, i.e., 10 days younger than in the first 2 experiments.

The fourth experiment was identical to the third except that caries-susceptible rats of our mutant albino strain were used.

At the termination of each experiment, the heads were preserved in 95% ethanol and then scored for carious lesions in the occlusal sulci by the method of Shaw et al. (9). This scoring method was not fully adaptable to smooth surfaces. The following procedure was developed to rep-

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

resent the frequency of attack and the extent of damage by the carious lesions. The mesial, lingual, distal and buccal surfaces were considered as 4 separate areas. Caries scores were assigned from zero to 3+. A value of 1+ represented an early demineralization of the enamel surface, 2+ included penetration into the dentin, and 3+ a major involvement of the enamel and dentin.

RESULTS

The results of experiments 1 and 2 are shown in table 2. The growth and development of the rats in the 2 control groups were typically normal for males and females of these strains fed cariogenic diet 2700. Growth in all the other 4 groups of both experiments was very poor by comparison. In groups 2 and 4 (diets 390V and 374V), growth for males was about one-third of the normal control values and for females about one-half. In groups 3 and 5 (diets 390C and 374C) where the diets contained 8% casein, growth was appreciably better than in the comparable diets 390V and 374V. Diet 374C produced slightly better growth than diet 390C. However, growth was still strikingly suboptimal even with these 2 diets, especially for the male rats.

The incidence of lesions in the occlusal sulci of the molars of the control rats fed diet 2700 was typical for both the Harvard and for the mutant albino caries-susceptible strains. When diets 390V and 390C were fed in groups 2 and 3 of the Harvard strain, highly significant reductions in the occurrence of carious lesions in the occlusal sulci were observed. However, when diets 390V and 390C were fed to groups 2 and 3 of the mutant albino strain, smaller reductions in the incidence of occlusal lesions were observed. The percentage reduction in group 2 was almost the same as for the Harvard strain but the difference was statistically significant at the $P < 0.05$ level instead of the $P < 0.01$ level. However, in group 3 of the mutant albino experiment the reduction was not statistically significant. When diets 374V and 374C were fed in groups 4 and 5 to both strains of rats, the incidence of occlusal lesions observed in both experiments was very similar to that of the control groups.

Smooth surface lesions were observed in 3 of the 20 control rats in group 1 of the Harvard strain. While the individual scores of these 3 rats were fairly high, the average score for the group was very low due to the small percentage of affected rats. In contrast, the occurrence of smooth surface lesions in the control group of the mutant albino experiment was appreciably higher, with 10 out of the 22 control rats developing smooth surface lesions. In each of groups 2 through 5 of the Harvard strain fed the whole wheat flour and white bread diets, significantly higher incidences of smooth surface lesions were observed. The highest frequency of affected animals and the highest average score were observed in group 2. The next highest values were observed in group 3 and the lowest increases in both frequency of affected rats and average score for smooth surface lesions were observed in groups 4 and 5 in which the results were very similar to each other. Similar but not identical results were obtained in the mutant albino experiment. In group 2, a highly significant increase in smooth surface lesions was observed. In group 3 an increase was noted which was statistically significant ($P < 0.05$). In groups 4 and 5, no increase in smooth surface lesions above those of the controls was observed. In comparison with experiment 1 where the the subjects were Harvard caries-susceptible rats, the behavior with respect to the development of smooth surface lesions was similar for groups 2 and 3. However, in groups 4 and 5, no increase was observed in experiment 2 in contrast to a significant increase in the first experiment.

The results of experiments 3 and 4 are presented in table 3. Slightly lower weight gains were observed in this experiment due to the experimental period being 10 days shorter. The rates of growth and development for the controls in group 1 of each experiment were normal. The supplement of 3% lysine and the increase in glucose monohydrate groups 3 and 4 did cause an improvement in the very slow rate of growth above that of group 2 in each experiment that diet 390V was fed. However, the incorporation of 18% skim milk powder in diets 590V and 591V caused significant improvements in the

TABLE 2

Influence of diets composed largely of whole wheat flour or of white bread on initiation and progression of carious lesions in occlusal sulci and on smooth surfaces [1]

Group no.	Diet	No. of rats	Wt gain		Carious lesions in occlusal sulci				Carious lesions on smooth surfaces			
			Males	Females	% Rats with caries	No. of carious molars	No. of carious sulci	Extent of carious lesions	% Rats with caries	No. of carious molars	No. of carious surfaces	Extent of carious lesions
			g	*g*								
					Experiment 1 — Harvard caries-susceptible rats							
1	2700	20	225.7 (8.7)	137.2 (2.5)	100	6.7 (0.4)	12.1 (1.1)	45.8+ (4.8+)	15	0.3 (0.1)	0.4 (0.2)	0.6+ (0.3+)
2	390V	23	79.5 [2] (5.1)	78.0 [2] (4.5)	91	4.4 [2] (0.5)	7.3 [2] (1.0)	26.8+ [2] (4.3+)	74	3.8 [2] (0.7)	5.7 [2] (1.0)	12.2+ [2] (2.1+)
3	390C	21	100.6 [2] (8.1)	84.8 [2] (2.4)	62	2.5 [2] (0.6)	3.5 [2] (0.8)	11.9+ [2] (2.7+)	67	2.8 [2] (0.6)	4.0 [2] (0.9)	8.5+ [2] (2.0+)
4	374V	21	84.8 [2] (6.8)	77.6 [2] (2.4)	100	6.8 (0.4)	12.8 (0.8)	49.5+ (3.5+)	48	1.5 [3] (0.4)	1.9 [3] (0.6)	4.2+ [3] (1.4+)
5	374C	20	132.0 [2] (8.5)	109.6 [2] (3.9)	100	7.5 (0.4)	13.9 (1.0)	53.6+ (4.5+)	40	1.9 [3] (0.7)	2.6 [3] (0.9)	5.7 [3] (2.0+)
					Experiment 2 — Mutant albino caries-susceptible rats							
1	2700	22	187.3 (10.8)	127.3 (4.5)	100	6.8 (0.4)	13.0 (1.1)	44.0+ (4.7+)	45	2.0 (0.5)	2.3 (0.6)	5.9+ (1.7+)
2	390V	22	56.6 [2] (8.3)	54.7 [2] (8.0)	91	5.2 [3] (0.6)	8.4 [3] (1.2)	26.0+ [3] (4.9+)	95	6.1 [2] (0.6)	8.0 [2] (1.1)	16.5+ [2] (2.6+)
3	390C	22	96.2 [2] (14.2)	87.5 [2] (10.4)	91	5.2 [3] (0.6)	9.2 [3] (1.3)	32.2+ (5.5+)	90	3.8 [3] (0.5)	4.6 [3] (0.8)	10.5+ [3] (2.0+)
4	374V	23	78.7 [2] (4.6)	75.6 [2] (4.7)	100	6.8 (0.4)	11.1 (0.9)	36.1+ (3.8+)	56	2.2 (0.5)	2.6 (0.6)	5.3+ (1.4+)
5	374C	21	128.5 [2] (5.8)	97.6 [2] (3.3)	95	6.9 (0.5)	10.7 (1.1)	33.0+ (3.5+)	67	2.7 (0.6)	3.4 (0.8)	6.5+ (1.6+)

[1] The upper value in each pair is the average while the lower value in parentheses is the standard error of the mean. Probability (P) values were determined for each average in an experimental group versus the average for the control group. Where no numeral follows the average for an experimental group, the difference from the control values was statistically insignificant.

[2] $P < 0.01$.

[3] $P < 0.05$.

TABLE 3

Influence of modifications of whole wheat flour diet on initiation and progression of carious lesions in occlusal sulci and on smooth surfaces [1]

Group no.	Diet	No. of rats	Wt gain		Carious lesions in occlusal sulci				Carious lesions on smooth surfaces			
			Males	Females	% Rats with caries	No. of carious molars	No. of carious sulci	Extent of carious lesions	% Rats with caries	No. of carious molars	No. of carious surfaces	Extent of carious lesions
			g	*g*								
				Experiment 3 — Harvard caries-susceptible rats								
1	2700	28	193.4 (8.8)	122.9 (3.8)	97	5.4 (0.4)	8.6 (0.9)	30.6+ (3.4+)	36	0.7 (0.2)	0.9 (0.4)	1.8+ (0.9+)
2	390V	25	63.8 [2] (9.1)	62.6 [2] (3.0)	80	3.2 [2] (0.5)	4.8 [2] (0.9)	16.7+ [2] (1.6+)	36	1.5 (0.5)	1.5 (0.5)	3.1+ (1.1+)
3	391V	25	66.7 [2] (6.9)	57.1 [2] (5.7)	89	5.3 (0.3)	8.4 (0.9)	28.1+ (3.3+)	52	2.0 [2] (0.4)	2.6 [3] (0.6)	6.0+ [3] (1.4+)
4	490V	25	59.6 [2] (5.3)	54.0 [2] (5.5)	100	2.7 [2] (0.4)	4.6 [2] (0.7)	15.5+ [2] (2.6+)	56	1.8 [3] (0.5)	2.4 [3] (0.5)	5.0+ [3] (1.3+)
5	590V	25	197.1 (7.1)	109.9 [3] (3.0)	92	4.3 [3] (0.3)	6.2 [3] (0.4)	19.4+ [3] (2.3+)	52	1.1 (0.2)	1.6 (0.4)	3.4+ (0.8+)
6	591V	25	175.0 (10.7)	122.3 (5.8)	100	5.9 (0.3)	9.6 (0.8)	31.0+ (2.8+)	60	1.6 [3] (0.3)	2.0 [3] (0.4)	4.5+ [3] (1.1+)
				Experiment 4 — Mutant albino caries-susceptible rats								
1	2700	25	182.9 (7.6)	119.7 (2.8)	100	5.9 (0.4)	11.7 (1.1)	31.7+ (4.1+)	32	1.6 (0.5)	1.6 (0.5)	3.6+ (1.1+)
2	390V	23	41.3 [2] (4.3)	42.8 [2] (6.2)	91	4.9 (0.6)	8.8 (1.3)	29.5+ (4.9+)	96	5.8 [2] (0.4)	6.9 [2] (0.6)	13.0+ [2] (1.4+)
3	391V	22	37.1 [2] (4.4)	35.1 [2] (5.1)	100	7.0 [3] (0.4)	12.8 (1.9)	44.9+ [3] (4.9+)	100	6.0 [2] (0.3)	6.6 [2] (0.5)	14.9+ [2] (1.4+)
4	490V	21	42.0 [2] (9.0)	44.7 [2] (3.1)	100	4.9 (0.5)	8.5 (1.2)	28.5+ (4.6+)	100	5.6 [2] (0.2)	6.5 [2] (0.5)	14.1+ [2] (1.5+)
5	590V	24	155.0 [3] (8.9)	115.4 (3.3)	100	6.0 (0.4)	12.3 (1.0)	40.0+ (4.0+)	83	4.2 [2] (0.4)	4.5 [2] (0.5)	9.2+ [2] (1.2+)
6	591V	24	171.4 (7.1)	110.5 [3] (3.4)	100	7.5 [2] (0.4)	15.1 [3] (0.9)	51.8+ [2] (3.6+)	79	4.0 [2] (0.5)	4.2 [2] (0.5)	9.5+ [2] (1.3+)

[1] The upper value in each pair is the average while the lower value in parentheses is the standard error of the mean. Probability (P) values were determined for each average in an experimental group versus the average for the control group. Where no numeral follows the average for an experimental group, the difference from the control values was statistically insignificant.

[2] $P < 0.01$.

[3] $P < 0.05$.

weight gain, with the normal values for group 1 being approached in all of the comparisons.

The occurrence of carious lesions in the occlusal sulci of the rats in group 1 of each experiment was typical for this strain for the experimental period of 60 days. In the Harvard rats of the third experiment, diets 390V, 490V and 590V in groups 2, 4 and 5 all caused significant reductions in the occurrence of sulcal lesions. In contrast, in the parallel mutant albino experiment, diets 390V and 490V caused slight reductions which were not of statistical significance, while diet 590V resulted in an incidence of sulcal lesions which was indistinguishable from that of the controls. In the Harvard experiment, when the concentration of glucose monohydrate was increased to 36% in diets 391V and 591V in groups 3 and 6, no difference in the scores of sulcal lesions was observed from those of the controls. Evidently the increased glucose concentration in this strain had been able to offset the reduced tendency of the whole wheat flour diets to produce this type of carious lesions. In the mutant albino experiment, the inclusion of the additional 18% glucose in diets 391V and 591V caused modest increases beyond that of the controls. In the case of diet 591V the increase was of statistical significance at the 5% level.

The incidence of smooth surface lesions in the control group in the third experiment using Harvard caries-susceptible rats was greater than for the control group of experiment 1, largely because a higher percentage of this control group developed lesions on the smooth surfaces. In the mutant albino experiment, the caries score for smooth surface lesions among the controls was slightly lower than for the controls in the previous experiment with this strain. In the Harvard experiment, all of the whole wheat flour diets caused an increased incidence of smooth surface lesions. A slightly increased incidence was observed in group 2 with diet 390V. This increase was not of statistical significance and was substantially less than in the first experiment. Diets 391V, 490V and 591V caused modest increases in smooth surface lesions which were statistically significant at the $P < 0.05$ level. However, in all cases the higher incidences of experiment 1 were not duplicated. The question arises whether the shorter time period of this experiment had not allowed ample opportunity for a fuller development of the smooth surface lesions. In the mutant albino experiment, the 5 whole wheat flour diets caused highly significant increases in the scores for smooth surface lesions. These values were highest and almost identical for diets 390V, 391V and 490V. Diet 391V with the increased level of glucose had had no detectable adverse influence on the initiation and progression of smooth surface lesions. The caries scores for smooth surface lesions produced by diets 590V and 591V were appreciably lower than for the above 3 diets. Evidently the inclusion of 18% skim milk powder had reduced the tendency to cause smooth surface lesions. Again the higher level of glucose in diet 591V did not have any adverse influence over the lower level in 590V.

DISCUSSION

The use of the several white bread and whole wheat flour diets in these experiments resulted routinely in definite increases in the incidence of lesions on the smooth surfaces in representatives of both the Harvard and mutant albino strains of rats beyond the levels produced by our cariogenic diet 2700. The reasons for these increments are not known. This study furthers the suspicion that the carious process, although basically the same in all locations on the teeth, may have somewhat different characteristics and requirements at different sites.

The occlusal sulci of the rat tend to trap food debris readily. In the presence of caries-producing organisms and suitable dietary components as the microbial substrate, the teeth of rats of caries-susceptible strains will manifest a high caries activity in the occlusal sulci. A wide variety of diets will support caries activity in this site if the criteria are met of a caries-susceptible host, appropriate microorganisms, fermentable carbohydrate and, in addition, particle size and physical nature of the dietary components which will permit passage into and retention in the sulci. Present data suggest that there may be less

requirement for the development of the typical plaque of microorganisms, etc., than is apparently required in human carious lesions. In fact, in examination of the sulci of rat molars under a dissecting microscope or in histological sections, there is much less evidence of a plaque or similar structures than would be expected from clinical studies. However, on the smooth surfaces of the molars of rats, there is no comparable anatomical characteristic which would trap food debris and microorganisms. Instead, the microorganisms must become established on an area which is relatively self-cleaning in comparison with the occlusal sulci and go on to develop a plaque under which the carious process can develop. In these studies on the initiation and progression of smooth surface lesions, material much more typical of human plaque was observed on the smooth tooth surfaces which developed carious lesions than we have seen previously in experimental animals. Why this should occur in the white bread and whole wheat flour diets is not known. Some of the components of these diets may have physical characteristics which enable them to adhere more readily to the smooth surfaces than the components of diet 2700. On the other hand, the cereal diets, in contrast with diet 2700, may support better growth and development of one or more microorganisms which produce gelatinous colonies enabling easier adherence on the smooth surfaces and plaque formation.

From the routine overall consideration of the average number of molar teeth with carious lesions in the occlusal sulci versus the average number of molar teeth with carious lesions on the smooth surfaces in individual groups of rats, the former value was higher in 21 of the 22 comparisons. The only exception was group 3 of the first experiment with Harvard rats where the lowest incidence of occlusal lesions of all 4 experiments was observed. In this exception to the general, the value for molars with carious lesions in the occlusal sulci was almost identical to the value for molars with carious lesions on the smooth surfaces. Strictly speaking, due to different scoring methods, exact comparisons cannot be made between the average number of occlusal sulci with carious lesions and the average number of smooth surfaces with carious lesions, nor between the average extent of caries score attributable to occlusal lesions and the average extent of caries score attributable to smooth surface lesions. While the 2 methods for scoring the 2 different types of lesions are not identical and, in fact, cannot be made identical due to the different characteristics of the lesions and the areas where they are initiated, the methods are sufficiently similar to justify the generalizations that smooth surface lesions had begun in fewer loci and progressed to involve less tooth substance at the termination of these experiments than the lesions in the occlusal sulci. This observation suggests that the lesions on the smooth surfaces of the molars may be initiated later than those in the occlusal sulci or that the lesions on the smooth surfaces may progress more slowly once initiated. The averages for groups are not helpful in distinguishing between these two possibilities. From observations in appropriate individual rats within all the groups, it appears that the former hypothesis of later initiation is more likely.

Many of the rats had extensive involvement of the occlusal sulci at the end of an experiment but no evidence of smooth surface involvement. The question could always be asked whether longer exposure to the diet would have resulted in the production of lesions on the smooth surfaces. Simple extension of the length of experimental period would not answer this question. With the usual rate of progression of carious lesions in the occlusal sulci, so much tooth substance is destroyed by the end of the current experimental period that the integrity of the smooth surfaces is being lost or has been lost from within before being attacked from without. In fact, in some rats at the end of these experiments such extensive destruction of the molar crowns had occurred that absolute certainty of the origin of the destructive process was impossible.

The averages for the incidence of smooth surface lesions in these experiments indicate that there is a somewhat greater tendency for this type of lesion to develop in the mutant albino strain than in the Harvard strain; however, these strains are

very similar with respect to the initiation and progression of carious lesions in the occlusal sulci. In both strains, the smooth surfaces of the mandibular molar teeth were much more likely to develop carious lesions than the comparable surfaces of the maxillary molars. In this respect the destruction of smooth surface lesions is closely parallel to that of the lesions in the occlusal sulci. A high degree of bilateral symmetry was noted for the smooth surface lesions, which is also typical for lesions in the occlusal sulci.

The average values for the incidence of smooth surface lesions obscure other differences between the 2 strains with respect to this type of carious lesion. In the mutant albino strain the lesions tended to be broad and diffuse and much more frequent on the buccal than on the lingual surfaces. In the Harvard strain the lesions were much more discrete sharply defined patches on the lingual surfaces, especially near or at the cementoenamel junction. In both strains the frequency of initiation on the mesial and distal surfaces was much less than on either the buccal or lingual surfaces. Despite the different sites and characteristics of the smooth surface lesions in the 2 strains, as well as somewhat different levels of proneness to develop smooth surface lesions, the dietary changes in the 2 sets of 2 experiments each, caused closely parallel results for the 2 strains.

One of the most noteworthy results in these experiments was the ability to reduce the incidence of carious lesions in the occlusal sulci and simultaneously increase the incidence of lesions on the smooth surfaces. This observation certainly gives credence to our original speculation at the beginning of the series of experiments that different dietary and nutritional aspects were involved in the etiology of lesions on the 2 sites. Another observation which needs further study is the different distribution of smooth surfaces lesions between the representatives of the Harvard strain and the mutant albino strain. Yet despite this distribution, the change in average incidence of smooth surface lesions with variations in dietary composition was parallel for the 2 strains.

Further studies are underway to define the nature of the dietary differences by modification of both diets 2700 and the 390 series. In addition, attempts will be made to compare and identify the microorganism in the lesions on the smooth surfaces and those in the lesions in the occlusal sulci.

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Effect of Biotin Deficiency on Escape and Avoidance Learning in Rats [1]

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ABSTRACT The effect of biotin deficiency on the ability of Sprague-Dawley rats to acquire conditioned avoidance and escape reactions was investigated. Avoidance learning was impaired with both 5.0-second and 2.0-second intervals between conditional and unconditional stimuli. Escape learning was not affected, suggesting that the defect in avoidance learning was not due to motor impairment. Behavioral and biochemical evidence suggests that effects of the deficiency on the central nervous system may be expected.

Although the general physiological effects of biotin deficiency in experimental animals have been well documented, the effect on central nervous system function has received little attention. Among the few papers in the literature dealing with neuropathological changes in biotin deficiency, there is lack of agreement between the different workers. Thus, Shaw and Phillips (1) found no degeneration of the spinal cord or the sciatic nerve in the biotin-deficient rat. The same authors reported that there were no neuropathological changes in chicks suffering from mild biotin-deficiency (2). No anatomical changes in the central nervous system of biotin-deficient rats were observed by Sullivan et al. (3). However, Tharanne (4) reported serious lesions in the peripheral sympathetic system during the course of alopecia caused by biotin deficiency. Biotin deficiency in the dairy calf was shown to result in paralysis of the hind quarters, which could be cured by biotin treatment (5). According to Pellegrino (6), biotin treatment inhibited a predisposition of the dog toward experimental epilepsy.

It can be expected that a neurological derangement could manifest itself in a change in the behavior of the experimental animal. However, there has been no investigation on the behavioral aspects of biotin-deficiency. The present study deals with selected aspects of behavior in the biotin-deficient rat and evidence has been presented for the first time to show that the learning capacity of the rat is impaired in biotin deficiency.

METHODS AND MATERIALS

The investigation reported here consisted of a series of 3 experiments, each concerned with the effects of biotin deprivation on selected aspects of rat behavior.

Animals. Weanling albino rats [3] (male, of the Sprague-Dawley strain) were used in the present study. The rats were housed in individual cages in air conditioned quarters. Biotin deficiency was produced as described earlier (7) by feeding a diet of the following composition (g/100 g): dextrose, 71; spray-dried egg white, 20; salt mixture no. 446 (8), 4; corn oil, 4; vitamin premix (biotin-free),[4] 1; and thiamin·HCl, 8 mg. Control rats received the same diet as the deficient animals and in addition received 2 injections of biotin (200 μg) intraperitoneally at the begin-

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[3] Camm Research Institute, Wayne, New Jersey.

[4] General Biochemicals, Chagrin Falls, Ohio; supplemented to the diet (1% level) to provide the following vitamins (mg/kg diet): vitamin A conc (200,000 USP units/g), 99.200; vitamin D conc (400,000 USP units/g), 5.511; α-tocopherol, 110.220; ascorbic acid, 992.000; *i*-inositol, 110.220; choline chloride, 1653.400; menadione, 49.603; *p*-aminobenzoic acid, 110.220; niacin, 99.200; riboflavin, 22.046; pyridoxine·HCl, 22.046; thiamine·HCl, 22.046; Ca pantothenate, 66.137; folic acid, 1.984; and vitamin B_{12}, 0.0298.

ning of the fourth and sixth weeks, and the biotin-deficient group received 2 injections of saline at the same time. All the rats were handled in precisely the same way. The rats were used for the experiment during the seventh and eighth weeks, by which time animals that did not receive biotin injections had become severely deficient.

Behavioral testing apparatus. The same basic apparatus was used in each of the 3 experiments and consisted of a grid-floor runway which has been described in detail previously (9). The apparatus constructed of plywood painted grey was 1.524 m long, 15.24 cm wide and 20.32 cm high throughout. It was divided into 3 portions: a start box (30.48 cm × 15.24 cm), a runway (91.44 cm × 15.24 cm) and a goal box (30.48 cm × 15.24 cm). The floor of the start box and runway consisted of brass rods (3.175 mm diameter) spaced 1.905 cm apart (center to center) mounted in Plexiglas attached to the plywood walls of the apparatus. The floor of the goal box was plywood. Between each section of the runway was a guillotine door controlled by overhead cords.

The grid floor of the start box and runway was electrified by a constant current stimulator [5] fed through a shock scanner [6] which delivered approximately 6 pulses of 1.5 ma shock/second to the grid floor. The shock onset was controlled by means of a decade interval timer [7] activated by means of a micro switch which was turned on whenever the guillotine door was raised. By varying the setting on the decade interval timer it was possible to activate the grid either immediately after the door was raised for escape learning or after several seconds' delay for avoidance learning.

In the avoidance learning studies the conditional stimuli (CS) consisted of 6 lights (6 volts) mounted 15.24 cm apart at a distance of 22.86 cm above the grid floor and a door buzzer mounted on the wall above the apparatus which provided a sound stimulus 10 db above the sound level of 71 db in the test room. The CS remained on during the period between the raising of the door and the onset of the unconditional stimulus (UCS) and until the animal escaped from the runway to the goal box at which time both the CS and the UCS (shock) were terminated by the animal passing through a photo electric cell [8] beamed across the entrance to the goal box. A standard electric timer controlled in the same manner as the shock and CS was used to record the length of time required for the animal to run from the start box to the goal box.

Testing procedure. Experiment 1 was concerned with the effects of the biotin deprivation on the animal's ability to learn to avoid an electric shock in the runway. Animals were given 5 trials/day for a total of 7 days. On each trial the animal was placed in the start box and the guillotine doors separating the start box from the runway and the runway from the goal box were raised. Raising the door initiated a 2.0 second "delay" period after which the UCS (shock) was switched on by means of an interval timer. If the animal reached the goal box before the shock onset the response was scored as an "avoidance;" if it failed to do so the response was scored as an "escape." Running time between the start box and goal box was recorded on each of the trials which were spaced approximately 3 minutes apart.

Experiment 2 involved avoidance learning as in experiment 1 except that the time interval between CS and UCS was extended from 2.0 seconds to 5.0 seconds so as to afford the animals greater opportunity to respond to the CS. A slight modification was made in the apparatus before running the last group of animals in the experiment. A second photo electric cell and timer were added to the circuit with the beam of the cell directed across the guillotine door separating the start box from the runway. This timer was turned on when the door was raised and went off when the animal broke the beam by leaving the start box, thus affording a measure of the length of time it took the animal to respond to the CS.

[5] Model 228, C. J. Applegate Co., Denver, Colorado.

[6] Model 1311 SS, Lehigh Valley Electronics, Fogelsville, Pennsylvania.

[7] Hunter Manufacturing Corporation, Iowa City, Iowa.

[8] See footnote 7.

The procedure in experiment 3 was identical to that in experiments 1 and 2 with the exception that there was no delay between the raising of the door (CS onset) and the onset of the shock (UCS). This then, was in effect an escape learning experiment designed to reveal the presence of possible motor effects of the dietary restrictions. With the use of the 2 photo cells and timers it was possible to record the length of time it took the animal to respond to the shock by leaving the start box (reaction time) as well as the length of time it took the animal to travel the length (91.44 cm) of the runway (running time).

The experimental design and sample sizes are summarized in table 1.

RESULTS

The results of experiment 1 indicate clearly that the biotin-deficient animals encountered considerable difficulty in avoiding the electric shock in the runway. Figure 1 shows the mean number of avoidance responses made by experimental and control animals as a function of training. The difference between the 2 groups (summed across trials) is significant ($P < 0.002$, White's Rank test (10)). Inspection of figure 1 indicates that neither group of animals made very many avoidance responses on the first 5 trials (day 1) of testing. This afforded us with an opportunity to compare the escape responses of the 2 groups. The mean escape time for each animal in trials 1 to 5 was therefore used to compare the 2 groups. The biotin-deprived group ($\bar{X} = 6.60$ seconds) was significantly slower ($P < 0.002$, Student's t test) than the normal group ($\bar{X} = 4.57$ seconds). The 2 groups could not be compared on

TABLE 1

Summary of experimental treatment of animals in experiments 1, 2 and 3

	Dietary treatment	Sample size	Behavioral testing
Exp. 1	Biotin-deficient	32	2.0-second avoidance
	Control	21	2.0-second avoidance
Exp. 2	Biotin-deficient	16	5.0-second avoidance
	Control	18	5.0-second avoidance
Exp. 3	Biotin-deficient	8	escape
	Control	10	escape

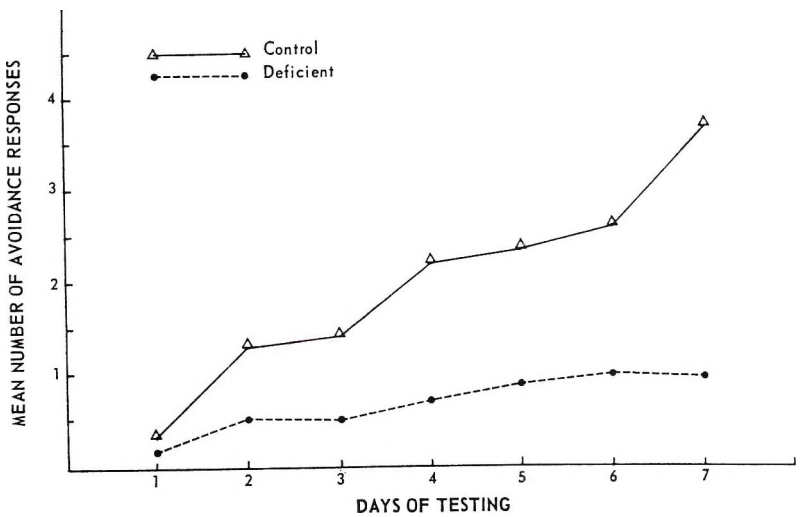

Fig. 1 Mean number of avoidance responses as a function of training on 2.0-second avoidance task.

this measure on subsequent trials since the normal animals began to avoid the shock and hence were making fewer escape responses than the experimental animals.

In experiment 2 the animals were allowed 5.0 seconds in which to avoid the shock. This resulted in a general improvement in the performance of both groups when compared with the subjects in experiment 1. Figure 2, which shows the mean number of avoidance responses as a function of training, indicates that at all stages of training the biotin-deprived animals made fewer avoidance responses. The difference between groups (summed across trials) is significant ($P < 0.002$, White's Rank test (10)). The failure of the experimental animals to learn the avoidance response as rapidly as the controls even when allowed more time to respond to the CS, suggested that perhaps the increased escape time in trials 1 to 5 in experiment 1 did not represent a slowness in running per se, but rather a failure of the animal to initiate a response to the CS as rapidly as the control animals. This hypothesis was confirmed by the use of the second photo electric cell placed across the exit from the start box for the last 8 deficient and the last 10 control rats. The length of time required for the animal to leave the start box and break the first photo electric beam is referred to as the reaction time. Mean reaction times (across the 35 trials) were calculated for each animal and the 2 groups compared. The mean reaction time for the control animals was 1.97 seconds and for the experimental animals, 4.38 seconds. The difference between the 2 groups was significant ($t = 3.392$, df $= 17$, $P < 0.01$). The failure of the experimental animals to respond as rapidly to the CS could be explained as a failure in conditioning or alternatively as a motor deficit which resulted in the biotin-deprived animals being incapable of responding as rapidly as the normal animals.

Experiment 3 provided an opportunity to detect the presence of a motor deficit since the animals were tested under escape conditions where the UCS was presented immediately after the guillotine doors were raised and the reaction of the animals was to the UCS (shock) not the CS. Two measures were obtained in this study; reaction time to the UCS and running time, i.e., the length of time it took the animal to move from the first photo electric cell to the second (placed at the entrance to the goal box).

Figure 3 shows the mean reaction time to shock as a function of training for the

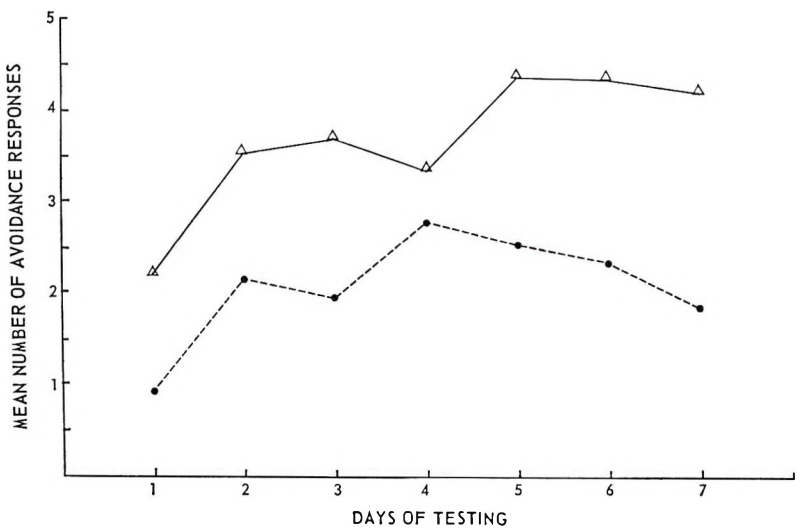

Fig. 2 Mean number of avoidance responses as a function of training on 5.0-second avoidance task.

2 groups of animals. The 2 groups were remarkably similar in their reaction times and statistical analysis of the difference between them (based on the mean reaction time over the 35 trials) reveals no significant difference ($t = 0.420$, df = 17, $P < 0.7$). The mean reaction time for the experimental groups was 0.90 seconds and for the control groups was 0.85.

Mean running times as a function of training for experimental and control animals are shown in figure 4. The mean running times over the 35 trials were 2.27 seconds for the deficient animals and 2.13 seconds for the control animals. This difference was not significant ($t = 0.604$, df = 17, $P < 0.6$).

The reaction time and running time data indicate that the biotin deprivation did not impair the motor ability of the animals to react to or escape from the UCS.

To rule out the possibility that the behavioral differences observed between the control and the biotin-deficient rats were due to the effects of inanition in the deficient rat, a control experiment was run in which pair-fed control and ad libitum-

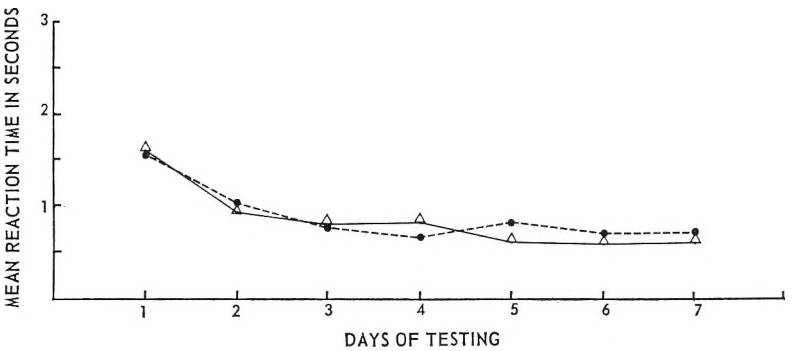

Fig. 3 Mean reaction time to shock as a function of training on escape learning task.

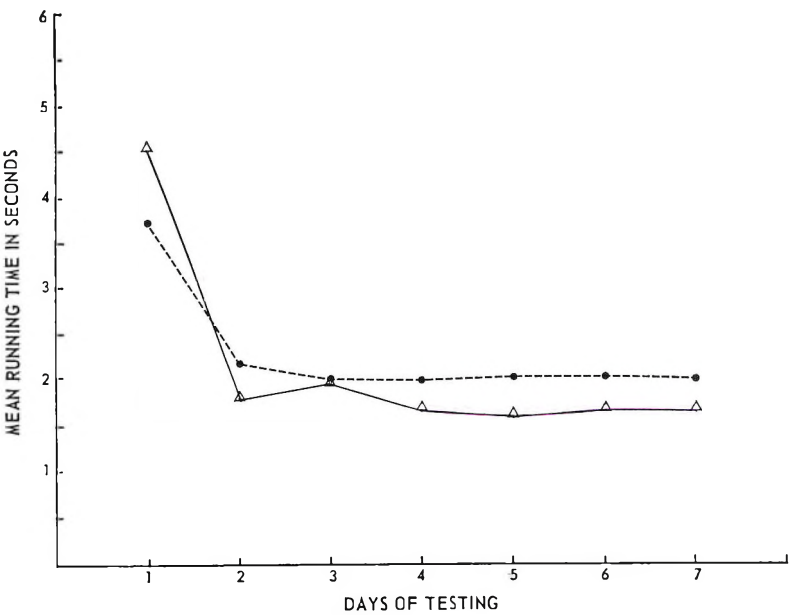

Fig. 4 Mean running time as a function of training in the shock escape situation.

fed control rats were tested (4 animals/group). No difference was observed between the pair-fed control and ad libitum-fed control rats in the number of avoidance responses (16.7 and 15.2, respectively, $P > 0.05$, White's Rank test (10)) in 35 trials of testing.

DISCUSSION

The results of experiments 1 and 2 suggest that biotin deficiency in the rat produces a deficit in the capacity of the animal to acquire a conditioned avoidance response. Experiment 3 which required the animals to simply escape from the electric shock provides rather conclusive evidence that the deficit in avoidance learning cannot be explained away as the result of a motor deficit. Reaction times to the UCS and running times to escape the UCS were essentially identical in the 2 groups of rats. The deficit in avoidance learning appears to result from a failure of the biotin-deficient animals to respond to the CS in the avoidance learning situation since reaction times to the CS were significantly greater for the deprived animals as shown in experiment 2. This deficit need not, of course, be interpreted as a deficit in learning per se, since it is conceivable that the biotin deficiency had produced a peripheral or central sensory deficit in the visual/or auditory systems, or in both of these. The use of a compound CS (visual, auditory plus vibration from raising the guillotine door), however, minimized the likelihood that degenerative changes, if any, in any one sensory system could account for the deficit. Thus far, there are no reports in the literature to indicate that neural or receptor degeneration results from biotin deficiency.

A motor syndrome in biotin-deficient rats has been reported (11) which was characterized by abnormality of locomotion, varying degrees of paralysis and spasticity. However, no explanation for the disturbance of locomotion could be offered by Lazere et al. (12) who did not observe any impaired neuromuscular relationships in biotin-deficient rats. They noted that such animals are sensitive to handling and suggested the reflex effects of painful stimuli upon the motor behavior in the deficient rats should be investigated. The results of our present investigation show no difference between the normal and the deficient rats in their response to a painful electric shock well above threshold for the rat. This does not, of course, rule out the possibility of an effect on the threshold for response to shocks or an effect on the qualitative nature of the response to shocks of low intensity. This problem is being investigated, using the jump-flinch technique of Kimble (13).

The present study suggests that the motor syndrome produced by biotin deficiency does not include a muscular weakness resulting in a quantitative deficit in running ability. It may be that this would appear at the extreme stages of deficiency but it is apparent that during the seventh and eighth weeks of biotin deprivation locomotive ability is normal even though the animals did show the peculiar gait described by previous workers.

The biochemical role of biotin in carbon dioxide fixation reactions and its mechanism of action have been well elucidated during the last few years (14). Among the indirect effects of biotin deficiency (15) are the following: (a) decreased utilization of glucose,[9] (b) decreased protein synthesis due to decreased sRNA synthesis (16) and a lowered P/O ratio in liver mitochondria.[10] A decrease in in vivo carbon dioxide fixation and in hepatic glycogen synthesis has also been reported (7). Most of these studies have been carried out in vivo or in vitro using liver preparations from experimental animals.

The present knowledge on the biochemical changes in the brain of the biotin-deficient rat is very meager. However, one could speculate on the effect of biotin-deficiency on brain metabolism on the basis of our knowledge of the metabolism in the intact animal or in tissue preparation in vitro. Brain accounts for a fifth of the total oxygen consumed by the body and under natural conditions respires almost excusively at the expense of glucose (17). Thus, an impairment in glucose utilization could result in a gen-

[9] Dakshinamurti, K., V. V. Modi and S. P. Mistry 1962 Carbohydrate metabolism in biotin deficiency. Federation Proc., 21: 468 (abstract).

[10] Modi, V. V., K. Dakshinamurti and S. P. Mistry 1962 Biotin deficiency and oxidative phosphorylation. Federation Proc., 21: 468 (abstract).

eral impairment in energy production. The efficient operation of the TCA cycle requires the synthesis of dicarboxylic acids which is known to be markedly reduced in biotin deficiency (15). Thus, "energy deficiency" could then be termed the net consequence of biotin deprivation in the experimental animal. According to Berl et al. (18), the operation of the carbon dioxide fixation mechanism in the brain is similar to that in the liver and hence, all such reactions dependent on biotin would be decreased in the brain of the biotin-deficient rat. Decreased protein synthesis in biotin-deficient rats due to decreased sRNA synthesis has been reported earlier (16). A moderate degree of protein deficiency in rats has been shown to lead to a decrease in cerebral enzymes and amino acids with associated behavioral deficit (19).

Although there are no reports in the literature on biochemical changes in the brain of the biotin-deficient animal to suggest changes in its behavior, it is apparent from the above discussion that such effects could be expected. To date no behavioral studies have been carried out with biotin-deficient animals. The data presented in this paper demonstrate clearly an impairment in selected aspects of behavior in the biotin-deficient rat and thus suggest a deleterious effect of the deficiency on central nervous system function.

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Effects of Levels of Protein and Gossypol, and Length of Feeding Period on the Accumulation of Gossypol in Tissues of Swine [1,2]

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ABSTRACT A study was made using swine to determine the effect of protein level, gossypol level, and the length of the feeding period on the accumulation of gossypol in the organs. The dietary levels were: protein, 14 and 28%; and gossypol, 0.03 and 0.06%. The periods of feeding were 14 and 28 days. The results showed that the levels of gossypol in the organs were inversely related to the dietary level of protein, and directly related to the level of gossypol in the diet and to the length of time the diets were fed.

Cottonseed meal contains the polyphenol, gossypol (1,1′, 6,6′, 7,7′-hexahydroxy-5,5′-diisopropyl - 3,3′-dimethyl-2,2′ binaphthalene-8.8′ dicarboxaldehyde). This compound, in the free state is toxic to nonruminant animals (1–5) when consumed in excessive amounts. Since swine cannot safely tolerate diets containing more than 0.01% free gossypol, the use of cottonseed meal as a source of protein for this species is restricted greatly (4). It has been shown, however, that high levels of dietary protein either alleviates or counteracts the toxic effects of gossypol in the diets of swine and of rats (4, 6, 7). The development of methods for the determination of free and of bound gossypol in tissues (8) provides means of studying factors affecting gossypol accumulation in the various organs of swine. Consequently, a study was made to determine the effect of different levels of protein and of gossypol on the accumulation of gossypol in the organs of swine subjected to these dietary treatments for different periods.

MATERIALS AND METHODS

The gossypol was prepared from cottonseed gums (9) by precipitation with aniline and pyridine as the dianilino-dipyridine derivative. This compound was converted to gossypol-acetic acid by hydrolysis with sulfuric acid in a mixture of ether and acetic acid (10). The gossypol-acetic acid was purified by recrystallization and was then converted to gossypol (10). The purity of the final product was 98%.

The corn was no. 2 grade, yellow, ground; and the soybean meal was solvent-extracted, 50% protein.

The diets were mixed in 18.2-kg batches. The basal diets, designated "gossypol-free," table 1, contained 14 and 28% protein, respectively, supplied by the soybean meal and corn. The "gossypol-supplemented" diets were the same as the gossypol-free diets except that 0.03 and 0.06% crystalline gossypol was added at each protein level. The gossypol was incorporated into the diets by grinding to a powder the calculated amounts (5.46 and 10.92 g/18.2 kg of diet) for the respective diets with a portion of the starch in a mechanical mortar. The gossypol-starch mixtures were then blended with the other dietary ingredients by means of a mechanical mixer.

From 9 litters, approximately 9 weeks of age, 28 Hampshire pigs averaging 22.7 kg in weight were used. From this group 3 pigs were assigned at random to each of the gossypol-supplemented diets for either the 14-day or 28-day feeding period. This

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TABLE 1

Composition of diets

	Dietary protein level	
	14%	28%
	%	%
Corn	40.00	40.00
Soybean meal	20.75	48.20
Starch [1]	20.50	5.00
Glucose monohydrate [2]	15.50	4.00
Dicalcium phosphate	2.00	1.00
Calcium carbonate	0.25	0.80
Trace mineral salt [3]	0.50	0.50
Vitamin antibiotic mix [4]	0.50	0.50
Total	100.00	100.00

[1] Gossypol was added at the rate of 5.46 and 10.92 g/18.2 kg of diet for the 0.03 and 0.06% gossypol-supplemented diets.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Composition of trace mineral salt: (in per cent) sodium chloride, 97.00; zinc, 0.800; manganese, 0.400; iron, 0.330; copper, 0.048; cobalt, 0.022; and iodine, 0.011.
[4] Vitamin and antibiotic supplement per kg of diet; (in IU) stabilized vitamin A palmitate, 2200; vitamin D, 1100; DL-α-tocopherol, 1.1; and (in milligrams) riboflavin, 4.4; pantothenic acid, 10.2; niacin, 22.0; choline chloride, 55.1; vitamin B_{12}, 0.022; and oxytetracycline, 0.022.

resulted in 4 groups of 3 pigs, individually fed, receiving gossypol-supplemented diets during each period, representing 2 levels of both gossypol and of protein per period. Due to limited facilities, only one pig was assigned to each of the gossypol-free diets per period. All pigs were fed at the rate of 5% of their body weight per day.

After 2 weeks, the pigs assigned to the dietary treatments for the 2-week period were killed; and the remainder were killed after the 4-week period had elapsed. Samples of blood were collected for the determination of hemoglobin content and for the preparation of blood serum. The body tissues were examined for gross lesions. The following organ tissues and body fluids were collected to be analyzed for free and for bound gossypol: liver, heart, lungs, spleen, kidneys, diaphragm muscle, pancreas, mesenteric lymph nodes, serum, and bile. The tissues were weighed and stored in plastic bags at −18° until removed for the preparation of the samples for the determination of dry matter, free and bound gossypol (8). The fluids were stored in glass containers. The samples, after preparation for analysis, were stored at −18° in screw-capped glass jars.

Statistical analysis of the data for the effects of protein level, gossypol level, and the length of feeding period on the accumulation of gossypol in the different organs was made according to a completely random design.

RESULTS AND DISCUSSION

The performance of the pigs was essentially normal; however, some of the pigs on either the high level of protein alone, or with added gossypol had slightly loose bowels. Food consumption was normal except for one pig fed the high gossypol-high protein diet, and that failed to consume the allowed amount for the last 5 days of the 28-day period. The average daily gain for the pigs fed the gossypol-free diets was 0.55 kg for each period, and was 0.50 and 0.55 kg for those fed the gossypol-supplemented diets for the 14 and the 28-day periods respectively. The hemoglobin ranged from 11.9 to 14.9 g/100 ml of blood for the pigs fed the gossypol-free diets and from 11.1 to 14.9 g/100 ml for those fed the gossypol-supplemented diets. These values are considered normal.

The amounts of free and of bound gossypol found in the various tissues are shown in table 2. Although a small amount of extraneous material was extracted and measured as gossypol for some of the tissues from the pigs fed the gossypol-free diets, the amounts were insignificant except for the "apparent free gossypol" in the diaphragm muscle of two of the pigs. The extracts from the diaphragm muscle of these 2 pigs had a pinkish tint which interferred with the spectrophotometric determination. All the tissues analyzed from animals fed the gossypol-supplemented diets contained both free and bound gossypol. Of the tissues analyzed, liver contained the highest level of both forms of gossypol, which observation is in accordance with results from a previous study (11). Free gossypol was higher than bound in the liver, bile, and diaphragm muscle, whereas the reverse was noted for the kidney, spleen, lymph nodes, lungs, and blood serum. The high level of bound gossypol in blood serum is in harmony with data of Lyman et al. (12) who reported that gossypol readily reacts with blood plasma albumin and cottonseed protein. The high level of free gossypol in the bile and the effect of free gossypol on the

TABLE 2

Mean gossypol content of organ tissues and body fluids of pigs

Tissue or fluid	Gossypol form	Gossypol content					
		14% Protein			28% Protein		
		Dietary gossypol, %			Dietary gossypol, %		
		0.00	0.03	0.06	0.00	0.03	0.06
		μg/g dry matter of tissue			*μg/g dry matter of tissue*		
		14-day Period					
Liver	F [1]	6.4	170.9	249.8	14.4	137.5	217.8
	B	3.2	133.3	234.7	7.2	94.1	165.1
Kidney	F	0	40.0	70.0	0	36.8	49.6
	B	0	49.5	108.4	0	35.8	69.4
Spleen	F	4.4	22.1	27.2	7.0	15.9	19.8
	B	6.5	38.9	68.0	8.2	25.8	48.3
Lymph nodes	F	3.6	7.6	37.7	3.6	10.6	31.5
	B	7.2	58.5	116.6	6.7	37.4	91.6
Lungs	F	0	12.7	26.3	0	13.1	30.6
	B	0	34.1	43.7	8.7	29.5	35.9
Heart	F	3.5	27.9	48.2	4.7	18.6	31.4
	B	4.3	23.3	38.5	6.2	23.0	33.8
Pancreas	F	0.8	16.5	26.3	0.3	10.3	16.5
	B	2.3	13.9	22.9	1.4	9.5	13.6
Diaphragm muscle	F	24.5	20.3	35.9	11.0	20.8	26.9
	B	9.1	24.5	29.4	8.4	14.3	20.0
Blood serum	F	0	4.7	15.3	0	20.5	25.8
	B	9.4	223.8	333.1	9.3	188.6	359.1
Bile	F	100.3	253.9	333.7	0	258.7	291.7
	B	9.5	35.1	43.2	11.5	53.5	43.4
		28-day Period					
Liver	F	3.2	241.6	322.6	9.7	140.6	269.8
	B	0	161.2	270.4	0	113.4	185.7
Kidney	F	0	48.8	77.7	0	41.7	71.9
	B	0	99.8	132.1	0	41.7	107.9
Spleen	F	6.4	32.0	38.1	4.8	29.8	37.0
	B	16.9	43.9	76.7	16.8	25.1	46.3
Lymph nodes	F	0.7	23.1	35.8	3.1	14.5	26.2
	B	5.8	69.2	141.9	7.5	48.6	82.8
Lungs	F	0	21.8	35.4	4.0	20.1	22.8
	B	7.7	38.5	56.4	4.0	31.3	40.3
Heart	F	3.9	38.2	52.9	6.0	23.5	45.6
	B	6.9	36.2	52.4	2.8	24.1	40.7
Pancreas	F	0.5	14.3	25.2	0.0	8.1	16.1
	B	1.0	18.0	26.3	3.0	9.5	20.0
Diaphragm muscle	F	12.2	24.8	34.9	19.3	18.6	30.9
	B	8.9	19.8	27.8	7.0	5.6	13.5
Blood serum	F	0	42.8	46.3	0	15.4	19.7
	B	6.3	299.4	378.8	4.7	170.5	301.3
Bile	F	67.4	290.6	440.7	92.4	349.0	273.0
	B	14.5	29.7	68.8	14.7	44.5	43.5

[1] F = free gossypol, B = bound gossypol.

gallbladder (13) suggest that bile is a medium for excretion of gossypol.

Statistical analysis of the data showed that the liver, heart, kidney, pancreas and diaphragm muscle from pigs fed the 28% protein gossypol-supplemented diets contained less free and bound gossypol ($P < 0.01$) than did corresponding organs from those pigs fed the 14% protein gossypol-supplemented diets. Similar differences ($P < 0.01$) were observed for the bound gossypol in the spleen, lymph nodes and lungs. Free gossypol in the spleens and lymph nodes of the 28% protein-fed pigs was lower than that for the corresponding organs of pigs fed the 14% protein diets ($P < 0.05$). A comparison of the effect of dietary gossypol showed that all organs

from the pigs consuming the lower level contained less of free and of bound gossypol ($P < 0.01$) than did corresponding organs from pigs fed the higher level. A similar difference was noted for bound gossypol in serum. Greater concentrations ($P < 0.01$) in free gossypol resulting from the longer periods of feeding were observed in the liver, kidney, heart, and spleen. Bound gossypol was higher for the longer feeding period in the liver and lungs ($P < 0.05$) and for the heart, kidney, pancreas and diaphragm muscle ($P < 0.01$).

The level of free and of bound gossypol observed in the organs of the pigs used in this study show an inverse relationship with the dietary level of protein. These observations are in agreement with and are suggested as an explanation of results reported by Hale and Lyman (6) that gossypol was fatal to pigs at the 0.03% dietary level when the diets contained 15% protein supplied by milo, cottonseed meal and soybean meal, but was not toxic when the dietary protein was increased to 30% by substituting additional soybean meal for milo. In a study with rats, Cabell and Earl (7) showed that the toxic effects of 9.5 mg of free gossypol per day could be nullified by an increase of 50% or more of a nitrogen intake of 160 mg/day supplied by either cottonseed meal or by a mixture of cottonseed meal and casein. They were not able to eliminate the effects of the 9.5 mg of gossypol by the addition, in proportions recommended by Steffie et al. (14), of 580 mg of essential amino acids to the cottonseed meal to supply a daily intake of 160 mg of nitrogen. Conkerton and Frampton (15) reported that the number of free ε-amino lysine groups of bovine serum albumen, egg albumen and cottonseed globulin was reduced when these products were reacted with gossypol. Baliga and Lyman (16) observed that gossypol reacted with purified cottonseed protein to form a complex, containing 3.25% bound gossypol, in which the apparent availability of the lysine was reduced from 82.9 to 48.7%. These workers (12) carried out additional studies on reactions of gossypol with protein.

Smith et al. (17) have shown that gossypol bound to cottonseed (ether-extracted) soybean or peanut meals lowered the growth-promoting properties of these products when fed to weanling rats in diets having protein as the limiting dietary ingredient. It was found that glandless (gossypol-free) cottonseed meal shaken for 30 minutes with gossypol dissolved in 0.1 N NaOH under an atmosphere of nitrogen contained 0.39% bound gossypol. Pure lysozyme reacted with gossypol, according to the procedure of Baliga and Lyman (16), contained 6% bound gossypol, and the product was highly resistant to digestion by rats and to pepsin and trypsin in vitro. The literature cited and experimental data presented strongly support the hypothesis that the high level of protein is responsible for the lower content of gossypol in the organs of the pigs fed the 28% protein diet, rather than an additional amount of some other component of the soybean meal. The high level of dietary protein may affect the accumulation of gossypol in the organs in 2 ways: (a) by supplying a greater number of free ε-amino groups with which the gossypol may combine in the digestive tract and then be excreted in the feces as bound gossypol, and (b) by facilitating the metabolism and detoxification of the absorbed gossypol. However, the use of very high levels of protein as a means of eliminating gossypol toxicity does not appear to be economically feasible.

Since this study was of short duration and the food consumption was normal, the gossypol in the organs varied with the level of gossypol in the diets as would be expected. Likewise, if gossypol is an accumulative poison, the amount stored in the organs should vary with the length of the ingestion period as was found.

The duration of this study was not sufficiently long for the development of severe symptoms of gossypol toxicity (13) but was adequate for determining the effects of dietary levels of protein and of gossypol and the period of ingestion upon the accumulation of gossypol in the organs. The greater portion of the accumulated gossypol was present in the liver. Approximately 40 to 45% of the liver gossypol was in the bound form, probably in combination with the free ε-amino groups of the proteins (12). It appears likely that liver being the site of much enzyme ac-

tivity, some of the metabolic functions of this organ may be impaired. Moreover, the high affinity of gossypol for iron may cause interferences with enzymatic functions involving iron. The distribution of ingested gossypol as the free or the bound form in the livers of pigs is affected by the injection of iron dextran intramuscularly (11). The high levels of free gossypol observed in the bile as confirmed by absorption spectra indicates that gossypol is eliminated from liver via the bile and hence may be either reabsorbed, combine with protein to be excreted as bound gossypol or as free gossypol. The latter is the probable explanation of the high levels of free gossypol in fecal samples (11). The high level of bound gossypol in blood serum may interfere with the oxygen-carrying capacity of the blood as suggested by Menual (18), thus putting an additional strain on the already over-taxed circulatory system as indicated by labored breathing, edema, and hypertrophy of the heart in the terminal stages of gossypol toxicity (13). It is apparent that many aspects of gossypol toxicity need further study.

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Chromium Deficiency in Rats: A Syndrome Simulating Diabetes Mellitus with Retarded Growth [1]

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ABSTRACT Glucose metabolism was studied in rats with moderate chromium deficiency induced by a diet low (0.17 μg/g) in the metal in an environment excluding airborne contaminants. Moderately hyperglycemic levels were observed in fasting serum of 83% of adult and 46% of young rats. Glycosuria occurred in 54.6% of chromium-deficient, and in 10.3% of chromium (III)-supplemented rats (2 ppm in drinking water). Females were more susceptible than males to these disturbances of glucose metabolism. Growth was moderately enhanced in chromium-fed animals; 30% of young rats of the third and fourth generations, bred without added chromium, grew slowly. Chromium-fed animals fed a diet of Torula yeast, sucrose and lard, deficient in vitamin E and with less chromium (0.1 μg/g), had a lower mortality, weighed more, showed less glycosuria but higher levels of serum glucose than did those deficient in chromium; hepatic degeneration was partly delayed. When this diet was supplemented with α-tocopherol, the differences in serum glucose, but not in other variables, remained. These data indicate the necessity of chromium (III) for normal glucose metabolism in rats.

Rats and mice given trivalent chromium in drinking water showed increased rates of growth, were larger at maturity (1, 2), lived longer and were less susceptible to an epidemic infection (3, 4), than controls. Rats exhibited virtually no spontaneous plaques in their aortas, unlike those not given chromium (5). These effects occurred with a diet marginally deficient in available chromium and in an environment as free of airborne chromium as possible. Chemical analyses of whole embryonic and newborn rats demonstrated chromium, whereas little or none was detectable in the livers of their mothers (6).

In 1959 Mertz and Schwarz (7, 8) showed that rats raised with certain commercial diets and with one composed of Torula yeast, sucrose and lard exhibited low tolerances to ingested or injected glucose. This defect was restored to normal by traces of chromium (III). Furthermore, as chromium (III) markedly enhanced the incorporation of glucose into fat by insulin, this trace metal was considered necessary for the action of insulin at the cellular level (9).

The rats studied by Mertz and Schwarz (7, 8) did not exhibit glycosuria or elevated fasting levels of glucose in serum, and their deficiency of chromium was considered marginal (10). Later, however, Mertz et al. studied the chromium-deficient rats raised by Schroeder et al. (4) for glucose uptake, finding that strict environmental conditions, to exclude metallic contaminants, enhanced a deficiency manifest by much more severe depression of glucose tolerance which was restored to normal by chromium (III) salts (10). Glycosuria also occurred in some animals after an oral dose of glucose (4). Therefore, glucose metabolism of such deficient rats was evaluated in an attempt to discover whether a disorder simulating diabetes mellitus in man could be produced in rats. Furthermore, as the rate of growth of young deficient rats appeared to be suppressed, the role of chromium as a trace element essential for optimal growth was re-evaluated.

MATERIAL AND METHODS

Rats of the Long-Evans strain were born in the low metal environment and raised with the low metal diet previously described (1–4); iron was added to food.

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Pregnant breeders obtained from the supplier [3] were isolated for one month in a separate room; electrostatically precipitated air under slight pressure was supplied to it and to the main laboratory, which is situated on a remote hilltop. The air intake was placed in a forest 80 m from the entrance to the laboratory to avoid dust and lead from motor vehicle exhausts.

The basic drinking water for all rats contained zinc, manganese, copper, cobalt and molybdenum (1); to it were added 2 ppm chromium (III) as the acetate for the controls. The basic diet of seed rye, dried skim milk and corn oil, with added vitamins, contained 0.17 μg/g chromium. A more deficient diet of Torula yeast [4] (30%), sucrose (59%) and vitamin E-free lard (10%) with salt mixture and vitamins added (1%), as recommended by Mertz and Schwarz (8), contained 0.1 μg/g chromium; it was deficient in vitamin E. Chromium appears to be concentrated in lipids (6); the preparation of α-tocopherol acetate [5] used had 0.39 ppm chromium, wet weight.

All animals except pregnant breeders were given the basic drinking water and the basic or Torula diet from the time of weaning. Chromium deficiency was induced as follows: 1) Nine male and 14 female rats were maintained with the basic diet until approximately 300 days of age, their mothers' having been fed the same diet without added chromium. 2) Twenty pregnant breeders obtained from the supplier were given the basic diet for 4 months ante- and postpartum. 3) Four generations of rats were bred from males and females fed the basic diet and maintained therewith without added chromium. 4) As part of another experiment, 24 female weanlings were given the basic diet and 5 ppm cadmium as the acetate in drinking water. Control animals were 52 male and 54 female rats fed the basic diet with 2 ppm chromium (III) added to drinking water and, as part of another experiment, 7 males and 10 females fed the basic diet and chromium were given cadmium, nickel, lead, 5 ppm each, in drinking water. In addition, 10 deficient males of the fourth generation were given the chromium supplement from the age of 120 to 134 days. 5) Male rats numbering 84, bred from animals not given chromium, were given the diet of Torula yeast; 36 were given supplements of α-tocopheryl acetate and 48 were not; 18 of each group were not fed chromium and the remainder were given 2 ppm in drinking water.

Glucose in serum was measured by a Berkeley Medical Instrument spectrophotometer, using glucose oxidase (11). Means of duplicate analyses were within 2%. Blood from animals fasting 18 hours was obtained from the tail of the warmed unanesthetized rat and centrifuged within one hour. Urine was assayed semi-quantitatively by test strips [6] on random samples from nonfasting rats; by this method glucose was estimated as 0.25%, 0.25 to 0.5% and more than 0.5%. Animals were weighed to the nearest 0.5 g at suitable intervals. Data were analyzed statistically by Student's *t* or the chi-square tests.

RESULTS

Basic diet, serum glucose. In table 1 are the fasting levels of serum glucose of mature rats with dietary chromium deficiency. Chromium-supplemented males exhibited higher values than did females, whereas deficient animals were similar in this respect. Differences in the means between supplemented and nonsupplemented groups were significant. Mean levels of rats given cadmium, lead and nickel, as well as chromium, deviated little from those of other chromium-fed animals. Postpartum females fed the deficient diet for a short time had less hyperglycemia than did those maintained with the diet from the time of weaning.

The upper limits of normal serum glucose were estimated at 3 times the standard errors of the means added to the means of the supplemented controls. In males this level was 122 and in females 115 mg/100 ml. Two deficient males had 125 and five, 136–170 mg/100 ml glucose in serum; two had "normal" levels. Of deficient females, 12 of 14 had 130 to 180 mg/100 ml and two, "normal" levels. Therefore, a mixed population apparently existed among the deficient animals. Of the 20 less deficient postpartum breeders, eight

[3] Blue Spruce Farms, Inc., Altamont, New York.
[4] Nutritional Biochemicals Corporation, Cleveland.
[5] See footnote 4.
[6] Combistix, Ames Company, Inc., Elkhart, Indiana.

TABLE 1

Fasting serum glucose levels of mature rats partly deficient in chromium, basic diet

	Chromium-fed			Chromium-deficient			P value [1]
	No. rats	Age	Glucose	No. rats	Age	Glucose	
		days	*mg/100 ml*		*days*	*mg/100 ml*	
			Males				
Mature	20	642	116.6 ± 1.7 [2]	9	300	137.2 ± 6.8	< 0.001
Same	11	718	106.5 ± 3.8				< 0.001
Cd, Pb, Ni [3]	7	509	110.1 ± 2.8				< 0.001
			Females				
Postpartum [4]				20	250	111.1 ± 3.9	< 0.025
Mature	21	645	95.6 ± 6.5	14 [5]	310	138.5 ± 5.7	< 0.001
Cd, Pb, Ni [3]	10	509	96.8 ± 8.8				< 0.001

[1] Significance of difference of chromium-fed and chromium-deficient groups.
[2] Mean ± SEM.
[3] Groups fed cadmium, lead and nickel (see text) for comparison.
[4] Breeders fed chromium-sufficient diet before pregnancy, bred once.
[5] Bred three or more times.

TABLE 2

Fasting serum glucose levels of young rats of third and fourth generations raised without chromium supplement

	Without glycosuria			With glycosuria			P value [1]
	No. rats	Age		No. rats	Age		
		days	*mg/100 ml*		*days*	*mg/100 ml*	
			Males				
Third generation	9	95	62.6 ± 6.9 [2]	9	94	104.2 ± 6.5	< 0.001
	5	120	92.2 ± 5.4				
Fourth generation	10	125	125.0 ± 4.1 [3]	12	120	120.0 ± 4.8 [4]	ns
				6	115	120.2 ± 4.0	ns
			Females				
Third generation	13	96	99.0 ± 7.2	13	95	125.1 ± 8.3 [5]	< 0.025
	18	138	93.4 ± 2.9	11	93	127.2 ± 4.0 [6]	< 0.001
Fourth generation	10	125	122.6 ± 3.3	8	127	115.8 ± 1.8	ns
Cadmium-fed				24	102	137.3 ± 3.0 [6]	< 0.001

[1] Significance of difference from comparable group without glycosuria.
[2] Mean ± SEM.
[3] Differs from mean of mature supplemented group, $P < 0.025$.
[4] When the rats were fed chromium supplement (2 ppm in water) for 13 days, mean level was 105.5 ± 3.5 mg/100 ml ($P < 0.025$).
[5] Differs from mean of mature supplemented group $P < 0.005$.
[6] Differs from mean of mature supplemented group $P < 0.001$.

were hyperglycemic according to these criteria, with only three having levels above 130 mg/100 ml.

Table 2 shows mean serum glucose levels in young rats of the third and fourth generations without chromium supplementation, divided as to whether or not glycosuria was found. Fasting values were higher in third generation animals with glycosuria than without; this difference was not evident in the fourth generation. Only the females showed significantly higher mean values than mature chromium-fed animals ($P < 0.005$). There were 20 of 51 males and 27 of 73 females with hyperglycemia according to standards for mature animals. Of the 24 deficient females given cadmium, 23 had glucose levels above 118 and 16 levels of 130 to 170 mg/100 ml.

Glycosuria. The number of rats exhibiting some degree of glycosuria is given in table 3. This observation was more frequent in young chromium-deficient animals than in older ones ($P < 0.025$). Only 9 of 87 supplemented animals had glucose

in random specimens of urine, whereas 101 of the 185 deficient rats showed it. Glycosuria, when present, was usually at the 0.25% level; in 14 cases it was greater.

Growth. The growth rates of rats marginally deficient in chromium are shown in table 4, compared with those partly reported in a previous study (2) given 5 ppm and a new series given 2 ppm. The differences are significant, 2 ppm chromium in drinking water providing the same increase in males as 5 ppm. Females given the higher doses of chromium weighed somewhat less than those given the lower dose. Male rats given supplements of chromium weighed 25.4% more at 60 days of age, 14.6% more at 90 days of age and 11.2% more at one year, than did chromium-deficient animals. Female rats given supplements of chromium weighed 20.1%, 13.4% and 12.1% more than deficient animals in these same intervals.

TABLE 3

Glucose in random specimens of urine of rats deficient in chromium [1]

	Chromium-fed			Chromium-deficient		
	No. rats	No. positive	% positive	No. rats	No. positive	% positive
			Males			
Mature	49	3		20	6	
Third generation				23	12	
Fourth generation				34	20	
Totals	49	3 [2]	6.1	77	38 [2]	49.4
			Females			
Mature	38	6		7	3	
Third generation				55	32	
Fourth generation				22	12	
Cadmium-fed				24	16	
Totals	38	6 [2]	15.8	108	63 [2]	58.2

[1] Ages of mature rats are shown in table 1; third and fourth generation rats were 90–120 days of age; cadmium-fed females were 102 days of age.
[2] Difference from chromium-fed group by chi-square analysis, $P < 0.0005$.

TABLE 4

Weight of rats fed chromium

Days of age	No Cr	P value [1]	2 ppm Cr	P value [2]	5 ppm Cr	P value [3]
	g		*g*		*g*	
			Males [4]			
60	144 ± 4.5 [5]	< 0.001	193 ± 7.9	~ 0.005	168 ± 3.5	< 0.001
90	221 ± 7.5	< 0.001	259 ± 8.3	ns [6]	261 ± 5.3	< 0.001
120	277 ± 9.1	< 0.005	314 ± 8.3	ns	301 ± 5.3	< 0.025
150	308 ± 8.4	< 0.005	347 ± 8.6	ns	333 ± 6.4	< 0.025
180	329 ± 6.3	< 0.001	365 ± 7.1	ns	359 ± 6.9	< 0.001
360	388 ± 8.2	< 0.005	437 ± 12.3	ns	441 ± 6.5	< 0.001
			Females [4]			
60	119 ± 3.9	< 0.001	149 ± 3.2	< 0.001	131 ± 3.8	< 0.025
90	167 ± 3.4	< 0.001	193 ± 4.0	ns	183 ± 4.9	ns
120	195 ± 3.5	< 0.001	222 ± 3.2	< 0.01	207 ± 5.0	< 0.01
150	209 ± 2.1	< 0.001	237 ± 3.2	< 0.025	220 ± 6.2	< 0.01
180	220 ± 2.9	< 0.001	248 ± 4.2	< 0.01	229 ± 5.4	ns
360	239 ± 3.9	< 0.001	272 ± 3.6	< 0.025	257 ± 5.9	~ 0.01

[1] Significance of difference, according to Student's *t*, between control and 2 ppm.
[2] Significance of difference between 2 ppm and 5 ppm.
[3] Significance of difference between control and 5 ppm.
[4] There were 52–54 rats in each of the 6 groups.
[5] Grams ± SEM.
[6] Not significant ($P > 0.05$).

Therefore, the proportionate retardation of growth of the deficient rats was greatest at young ages.

Slow initial rates of growth were also observed among some rats of the third and fourth generations raised without chromium. At 60 days of age, 13 of 36 males and 25 of 91 females weighed 110 to 120 g, compared with 193 ± 7.9 for males and 149 ± 3.2 for females supplemented with chromium. Growth then became more rapid.[7] Chromium-deficient male rats showing glycosuria weighed 185 ± 6.7 g at 90 days and 208 ± 8.7 g at 102 days of age, compared with 197 ± 9.0 and 261 ± 11.5, respectively, for those without glycosuria (102 days, $P < 0.005$). Differences in weights between females with and without glycosuria, however, were not significant, although they weighed less than did chromium-fed animals ($P < 0.05$ to < 0.005).

Longevity and mortality. Some data additional to that previously reported (4) has accumulated on longevity of chromium-fed rats. The two oldest males died at the ages of 1344 and 1455 days,[8] compared with the two longest-lived deficient animals, at 1185 and 1154 days of age. Mean ages at death of the five oldest chromium-fed males (of 52) was 1281 ± 54 days; of the oldest deficient males (of 52) 1141 ± 14 days ($P < 0.025$), a mean difference of 4.7 months, or an increase of 12.3% in the chromium-fed group. Mean ages of the five oldest females of each group at death were 1293 ± 14 and 1292 ± 42 days, respectively. Median life-spans of these rats, however, were not affected by chromium (4). The degree of chromium deficiency produced by the basic diet also did not affect the incidence of cannibalism of dead animals (17% vs. 9% for chromium-fed rats), nor did it influence the incidences of mortality during the first year of life, which were negligible.

[7] This phenomenon was also observed previously (2), at a time when we were not aware that the basic diet was marginally deficient in chromium. Young male rats grew 80.7% as rapidly in the first 2 months of life as did those given chromium; young females 87.4%. During the next 2 months rates slightly exceeded those of chromium-fed animals.

[8] To our knowledge, no laboratory rat has been reported to have lived to this age, just short of 4 years.

TABLE 5

Effects of chromium on male rats receiving Torula yeast diet with and without added vitamin E

	Chromium-supplemented		Chromium-deficient		P value [1]
	No. tested or surviving	Variable	No. tested or surviving	Variable	
Vitamin E-deficient group					
No. of rats	30		18		
Serum glucose, mg/100 ml	19	108.9 ± 4.0	4	84.0 ± 13.9	< 0.02
Glycosuria, %, 60 days	18	5.5	10	80	< 0.0005
Weight, g, 60 days	30	109.6 ± 5.1	11	93.0 ± 3.2	< 0.05
90 days	22	134.0 ± 9.0	4	93.0 ± 3.0	< 0.025
120 days	18	139.9 ± 9.0	1	86.0	< 0.005
Mortality, %, 120 days		40.0		94.4	< 0.001
Vitamin E-supplemented group					
No. of rats	18		18		
Serum glucose, mg/100 ml	12	95.8 ± 3.8	12	72.5 ± 4.5	< 0.001
Glycosuria, %, 60 days	18	83.3	16	93.8	ns [2]
Weight, g, 60 days	18	77.2 ± 3.9	16	80.7 ± 2.2	ns
90 days	18	106.8 ± 4.1	16	110.3 ± 2.7	ns
120 days	18	131.2 ± 5.0	16	133.7 ± 2.1	ns
Mortality, %, 120 days		0		11.1	ns

[1] Significance of difference between the 2 groups.
[2] Not significant.

Torula diet. Some effects of chromium supplementation on male rats receiving the diet of Torula yeast, lard and sucrose are indicated in table 5. The rats deficient in both vitamin E and chromium exhibited markedly delayed growth, low serum glucose levels, glycosuria and a high mortality, all of 18 having died at 128 days, 17 at 120 days, 14 at 90 days and 7 at 60 days of age. Those supplemented with chromium grew significantly better, had little glycosuria, higher serum glucose levels and less mortality, all of 30 being alive at 60 days, 22 at 90 days, 18 at 120 days and 12 at 225 days of age ($P < 0.001$). Five of 19 of these rats were hyperglycemic. At death all animals showed varying degrees of hepatic degeneration which was less severe in the chromium-fed group.

When the diet was supplemented with vitamin E, effects of chromium on body weight, glycosuria and mortality were not evident. Growth rates, although depressed, were similar in both groups. Differences in glucose metabolism, however, appeared, the chromium-deficient animals having lower levels than the chromium-supplemented.

DISCUSSION

When precautions were taken to exclude airborne chromium from the animal quarters, and rats were given a diet low in chromium from the time of weaning, hyperglycemia and glycosuria appeared in a majority of mature animals, associated with some retardation of growth. The degree of chromium deficiency produced by this regimen, however, was moderate. The highest serum glucose levels were 170 to 180 mg/100 ml, and the disturbance of glucose metabolism could hardly be termed "severe." Lesser degrees of hyperglycemia occurred in mature pregnant females obtained elsewhere, given the low chromium diet for shorter periods and tested postpartum; presumably they had been raised with an adequate diet. Male rats were less susceptible to abnormal glucose metabolism induced by diet than were females.

Based on the criteria of hyperglycemia, glycosuria and retarded growth, some young rats raised for 4 generations in our laboratory without chromium supplementation appeared to have a more severe degree of deficiency, especially those exhibiting glycosuria. Hyperglycemia, however, was not as pronounced in the males as in the females, although retardation of growth was greater.

An attempt to produce greater degrees of chromium deficiency by giving male rats a diet of Torula yeast, sucrose and vitamin E-free lard, resulted in stunted growth, whether or not vitamin E was added. When hepatic degeneration or necrosis secondary to vitamin-E deficiency was induced, glycosuria without hyperglycemia occurred in the chromium-deficient animals, associated with cessation of growth and a high mortality. In them disturbances of glucose metabolism were probably consequent to the severe disease of the liver present. When vitamin E-deficient animals were given chromium (III), however, growth continued slowly, there was little glycosuria, serum glucose levels were higher, mortality was considerably lower and livers, although grossly abnormal, were less seriously affected. Therefore, the relationship of chromium (III) to vitamin E deserves study. Male rats supplemented with vitamin E exhibited glycosuria whether or not chromium was fed, possibly as a result of the sucrose in the diet. The low serum glucose levels in those deficient in chromium are unexplained. This diet was undoubtedly deficient in other nutrients, possibly sulfur-containing amino acids, as judged by the retarded rates of growth.

That chromium is a factor for increased growth rates for mice (1) and rats (2) is confirmed by the data here presented. In mice unpublished observations on a second series have shown consistent and significant ($P < 0.005$) increases in weights of animals given chromium, from 7.7% at 60 days of age to 21.3% at 360 days of age. That some of the tissues of deficient animals were low in chromium by chemical analyses has been reported (3, 4). The characteristic renal lesions of human diabetes mellitus, however, have not been detected on microscopic examination of tissues.

The evidence that chromium (III) may be an essential trace metal for rats is based on marginal or moderate states of deficiency. To this point, a diet completely

free of chromium, but containing all of the other necessary dietary factors, has not been found. Although the strictest criteria for essentiality of a micronutrient, failure of growth and survival of totally deficient animals, have not been fulfilled, the difficulty of preparing a diet free of this ubiquitous lipotropic metal may retard this experiment for some time.

To my knowledge, the present experiments are the first to demonstrate in rats a disturbed glucose metabolism simulating that of mild adult-onset diabetes mellitus of man as a deficiency disease.[9] Applications to the human disorder are warranted.[10]

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