Plasma Amino Acids and Nitrogen Retention by Steers Fed Purified Diets Containing Urea or Isolated Soy Protein

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ABSTRACT The nitrogen metabolism of steers fed purified diets containing either urea or isolated soy protein as the sole sources of dietary nitrogen was studied. Replacing glucose monohydrate with additional starch significantly altered fecal and urinary nitrogen losses and also the ratio of the ruminal volatile fatty acids. Nitrogen retention was significantly greater when the isolated soy diets were fed. Serine and glycine were detected in significantly greater quantities, whereas valine, isoleucine, leucine, and phenylalanine were detected in significantly smaller quantities in the blood plasma when steers were fed the urea diets. Evidence indicates that the lack of sufficient quantities of the branched-chain volatile fatty acids at the ruminal level may have been responsible for the lowered plasma levels of valine, isoleucine, and leucine when steers were fed the urea diets. These data also suggest that the lowered nitrogen retention may have been the result of a deficiency of these amino acids and phenylalanine or an imbalance of certain amino acids synthesize by ruminal microorganisms.

Ruminants, through the action of the microorganisms in the rumen, convert non-protein nitrogen into microbial protein which becomes available to the host animal. It has been shown with purified diets that ruminants may obtain all of their dietary nitrogen from urea (1, 2) or urea and ammonium salts (3) and obtain satisfactory but reduced performance. Growth under ad libitum-fed conditions is usually about 70% of that compared with the feeding of purified diets containing natural protein (2). Since all of the amino acids must be synthesized for a diet of this type, it seems logical that a deficiency or imbalance of one or more of the amino acids may be responsible for lowered performance. Loosli et al. (1) determined the amino acid content of ruminal ingesta, urine, and feces of sheep and goats fed a purified diet containing urea as the only dietary source of nitrogen and reported that the essential amino acids were synthesized in various amounts. Further information could be obtained by relating plasma amino acids to nitrogen retention.

The present experiment was conducted to study the nitrogen metabolism of steers fed purified diets which contained, as variables, either isolated soy protein or urea and either starch or starch and glucose monohydrate.[1]

EXPERIMENTAL PROCEDURE

The experiment was designed as a 4×4 Latin square with a 2^2 factorial arrangement of treatments. Angus steer calves averaging 208 kg were fed a daily amount of feed equal to 1.25% of each steer's body weight. Equal amounts of the feed (table 1) were offered at 8 AM and 4 PM and the feed was always consumed in less than 30 minutes. A metabolism trial was conducted using a 28-day adjustment followed by a 7-day collection period for each of the 4 periods. Feed, feces, and urine were analyzed for dry matter (except urine), ash, nitrogen (4) and acid detergent fiber (5).

Ruminal samples were obtained from each steer by stomach tube at the end of each collection period. Each sample was taken 2 hours after the morning diet was consumed and consisted of at least 1 liter of fluid. Ammonia (6), volatile fatty acids

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

TABLE 1

Composition and analysis of experimental diets

	Isolated soy		Urea	
	Glucose [1] + starch	Starch	Glucose + starch	Starch
	%	%	%	%
Cornstarch	23.3	46.6	28.4	56.8
Glucose [1]	23.3	0.0	28.4	0.0
Wood pulp [2]	30.0	30.0	30.0	30.0
Isolated soy protein [2]	14.9	14.9	0.0	0.0
Urea [3]	0.0	0.0	4.7	4.7
Mineral mix [2]	6.4	6.4	6.4	6.4
Refined soybean oil	2.0	2.0	2.0	2.0
Choline chloride	0.1	0.1	0.1	0.1
Vitamins [2]	+	+	+	+
Chemical analysis, % dry matter				
Crude protein	13.5	13.4	13.8	13.9
Acid detergent fiber	28.7	29.0	30.0	29.0
Ash	6.3	6.5	6.5	6.3

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Described by Oltjen et al. (9).
[3] Feed grade.

(VFA) (7) and pH determinations were made on the strained ruminal fluid.

Jugular blood samples (heparinized) were obtained from each steer 16 hours after the evening diet was consumed. These were taken at 8 AM on the day following the sampling of the ruminal ingesta and after the steers had consumed the test diets for 37 days. Ten milliliters of plasma were deproteinized in a glass-stoppered flask by the addition of 50 ml of 1% picric acid solution. After the mixture was shaken for a few seconds, it was centrifuged and 50 ml of the resulting supernatant were passed through a column of Dowex 2-X8 resin (200–400 mesh) in chloride form. The colorless effluent and washings were concentrated on a rotary evaporator to a volume of about 1 ml and then transferred into a 5-ml volumetric flask with the aid of 2 ml distilled water. This solution was adjusted to pH 7 to 8 by the drop-by-drop addition of 1 N NaOH and allowed to stand at room temperature for 4 hours to convert cysteine to cystine. Then the solution was adjusted to pH 2.0 by adding 1 N HCl and the volume increased to 5 ml by pH 2.2 citrate buffer. The samples were frozen until analysis was performed. The amounts of the individual amino acids and other ninhydrin-positive compounds were determined by ion exchange chromatography on an automatic amino acid analyzer (8).

RESULTS AND DISCUSSION

The results of the metabolism trial and ruminal fluid analysis are presented in table 2. Digestible dry matter and gross energy were similar across treatments. Digestibility of the wood pulp appeared to be depressed when glucose was replaced by starch but the difference was not statistically significant. Digestibility values for the acid detergent fiber agree closely with previous work with the urea diet but were about 10% lower than values obtained when isolated soy protein was previously used (9). The reason for this is not readily apparent.

Fecal nitrogen losses were greatest ($P < 0.05$) when starch and glucose were included in the diets. Urinary nitrogen losses were greatest ($P < 0.05$) when starch replaced glucose and when urea was the nitrogen source ($P < 0.01$). The beneficial effect of the lowered fecal nitrogen loss with the high starch diets was nullified by the greater urinary loss and, therefore, nitrogen retention values were similar for carbohydrate sources. Nitrogen retention was greatest ($P < 0.01$) when isolated soy was part of the diet. Nitrogen retention with urea diets was

TABLE 2

Apparent digestibility, nitrogen balance and ruminal data of steers fed the experimental diets

Criteria [1]	Isolated soy		Urea		Significant differences
	Glucose [2] + starch	Starch	Glucose + starch	Starch	
	Digestibility, % intake				
Dry matter	74.9	74.4	74.7	75.6	
Gross energy	71.9	71.3	71.3	72.7	
Acid detergent fiber	53.8	50.3	57.4	52.1	
	Nitrogen, % intake				
Fecal	30.6	28.3	28.8	22.6	2,4 < 1,3 [3]
Urinary	42.3	47.1	56.0	61.4	1,2 < 3,4 [3]
					1,3 < 2,4 [3]
Retention, %	27.1	24.6	15.2	16.0	3,4 < 1,2 [4]
Retention, g/day	13.7	11.3	7.8	8.1	3,4 < 1,2 [4]
	Ruminal data				
Mg NH_3-N/100 ml fluid	14.0	7.0	44.8	38.6	1,2 < 3,4 [4]
pH	6.0	5.8	6.2	6.5	
Volatile fatty acids					
Acetic, M%	58.0	61.2	50.3	62.1	1,3 < 2,4 [4]
Propionic, M%	25.8	25.8	27.7	24.8	
Butyric +, M%	16.2	13.0	22.0	13.1	2,4 < 1,3 [4]
Total conc, mmole/liter	92.9	93.3	109.0	84.2	

[1] Each value is an average of 4 steers.
[2] Cerelose, Corn Products Company.
[3] $P < 0.05$.
[4] $P < 0.01$.

64% of that when the isolated soy diets were fed.

During the metabolism trial, there was a decrease in urinary nitrogen with a consequent increase in nitrogen retention with period ($P < 0.01$). This was true for both nitrogen sources and may suggest an adaptation to the purified diet constituents. McLaren (10) has reported an adaptation to non-protein nitrogen by lambs; however, in the present trial the adaptation seemed apparent with both nitrogen sources.

Ruminal ammonia concentration was less ($P < 0.01$) when the steers were fed the isolated soy diets. There was also a trend for lowered ruminal ammonia values when starch replaced glucose in the diets. There is usually a positive relationship between ruminal ammonia and urinary nitrogen but in this study the lower ammonia values are associated with the higher urinary losses when carbohydrate sources are considered. The single ruminal sampling time may not be indicative of the ruminal ammonia pattern. Hoshino (11) reported that glucose was more effective than starch in lowering the ammonia liberated from casein in vitro, but in vivo, the opposite trend has been reported (12). Further indication of a different ruminal fermentation was the increase ($P < 0.01$) in the molar percentage of acetic acid, whereas the molar percentage of butyric plus higher acids decreased ($P < 0.01$) when starch replaced glucose in the diets.

The concentrations of the free amino acids in the plasma are shown in table 3. The mean squares of amino acids showing significant differences are shown in table 4. When the steers consumed the urea diets both serine ($P < 0.01$) and glycine ($P < 0.01$) were found in greater concentrations, whereas valine ($P < 0.01$), isoleucine ($P < 0.05$), leucine ($P < 0.01$) and phenylalanine ($P < 0.01$) were found in lower concentrations than when the steers consumed the isolated soy diets. Also the concentration of valine ($P < 0.05$) and isoleucine ($P < 0.05$) was greater when starch replaced the glucose in the diets. An interaction ($P < 0.05$) was detected for serine with greatest plasma concentrations observed for starch with isolated soy protein and urea with both starch and glucose.

TABLE 3

Free amino acids in the plasma of steers fed the experimental diets

Criteria [1]	Isolated soy		Urea	
	Glucose [2] + starch	Starch	Glucose + starch	Starch
	μmole/100 ml			
Lysine	6.8	5.1	5.3	7.2
Histidine	3.0	2.8	3.5	3.1
Tryptophan	0.7	0.7	0.4	0.8
Arginine	5.4	4.7	4.4	5.4
Aspartic acid	0.5	0.5	0.3	0.4
Threonine	2.7	3.1	2.4	3.4
Serine	3.6	4.7	5.9	4.9
Proline	3.4	4.1	3.7	3.5
Glutamic acid	3.7	6.0	5.6	4.1
Glycine	18.2	22.3	30.5	27.0
Alanine	12.5	15.4	14.0	11.5
Valine	14.7	18.9	11.6	12.3
Cystine	1.2	1.3	1.0	1.1
Methionine	1.0	1.0	1.0	1.2
Isoleucine	6.3	7.4	5.3	6.0
Leucine	6.9	8.3	5.5	5.1
Tyrosine	1.5	1.8	1.4	1.4
Phenylalanine	2.4	2.6	2.0	1.0
Total	94.5	110.7	103.8	99.4

[1] Each value is an average of 4 steers.
[2] Cerelose, Corn Products Company.

Total concentration of the amino acids was similar for both nitrogen sources and by measuring this alone it would be possible to conclude that both nitrogen sources had values similar to those of the steers. However, the individual differences show possible reasons why nitrogen retention was less ($P < 0.01$) with the urea diets. By assigning a value of 100 to each plasma amino acid concentration when the steers consumed the isolated soy diets, the following relative percentages were calculated when the steers were fed the urea diets: serine, 130; glycine, 142; valine, 71; isoleucine, 82; leucine, 70; and phenylalanine, 76. Growth data with sheep fed urea diets were about 70% of that of sheep fed isolated soy diets (2). Previous attempts to improve the performance of ruminants fed the urea diet by use of dietary additions of methionine, lysine, alanine, or glutamic acid have been unsuccessful (2, 9, 13).

The amino acid concentrations reported in table 3 are somewhat lower than those reported for lactating cows fed natural diets (14). However, the cows were given a large amount of feed and the blood samples were taken shortly after feeding. The total amino acid concentrations in the present study are similar to those determined for sheep fed natural diets when sampled just prior to feeding (15).

Duncan et al. (16) compared by microbiological assays the mixed ruminal proteins of calves fed a purified diet, in which urea supplied the dietary nitrogen, with those of a calf fed a natural diet. With the exception of histidine which was lower with the urea purified diet, the amino acid pattern was similar. In other studies when cows were fed a purified diet containing ^{15}N-labeled urea, histidine had the lowest labeling of all the amino acids in milk (3). In the present experiment the amount of histidine present in the plasma was similar for both purified diets. However, other amino acid analyses conducted in our laboratory indicate that histidine may be lower in the plasma of cattle fed purified diets than in those fed natural diets.

The nutritional significance of the elevated levels of glycine and serine in the plasma when the steers were fed the urea diets is obscure. However, it has been shown that the rate of ammonia excretion in acidotic dogs is increased by glycine (17).

TABLE 4

Mean squares showing significant differences

Source of variation	df	Amino acids						Ninhydrin-positive compounds			
		Serine	Glycine	Valine	Isoleucine	Leucine	Phenylalanine	Taurine	Hydroxyproline	Glutamine + asparagine	Citrulline
Nitrogen source (N)	1	6.38 [1]	289.00 [1]	93.12 [1]	5.76 [2]	20.70 [1]	1.38 [1]	0.25	0.39 [2]	49.35 [2]	0.95
Carbohydrate source (C)	1	0.05	0.30	24.50 [2]	3.06 [2]	1.10	0.02	0.64 [2]	0.10	35.70 [2]	0.33
N × C	1	4.52 [2]	57.00	12.25	0.09	3.42	0.11	0.16	0.23 [2]	14.25	8.56 [2]
Error	6	0.42	14.98	3.11	0.44	1.22	0.10	0.05	0.03	4.00	0.85

[1] $P < 0.01$.
[2] $P < 0.05$.

Allison and Bryant (18) using labeled materials have shown that in the rumen a significant quantity of microbial branched-chain amino acids may be synthesized from branched-chain fatty acids. It has also been shown that the branched-chain VFA's are produced from the catabolism of amino acids in the rumen (18–20). Other evidence indicates that the 4- and 5-carbon branched- and straight-chain VFA's are essential nutrients for the growth of some cellulolytic microorganisms of the rumen in vitro (19). Therefore, if these VFA's are not supplied in the diets as branched-chain amino acids, they must be synthesized in the rumen and aminated to the resulting amino acids. In the present experiment the iso- acids were not separated from butyric acid. However, Ørskov [2] has determined with gas-liquid chromatography that the ruminal ingesta of cows fed the present urea diet are either devoid of the branched-chain VFA's or they were detected in extremely small quantities. Matrone et al. (21) compared purified diets containing primarily urea or casein as nitrogen sources. They reported that isovaleric acid was detected in significantly smaller amounts in the ruminal contents of sheep fed the urea diet but isobutyric acid was not determined. Recently, Cline [3] reported a significant increase in the digestibility and retention of nitrogen when lambs were fed urea-containing purified diets supplemented with isobutyric, isovaleric and *n*-valeric acids. The addition of the acids decreased ruminal ammonia levels and suggested that more ammonia was being utilized within the rumen. Thus, it appears that in the present experiment insufficient quantities of the branched-chain VFA's in the rumen could have been responsible for the low plasma concentrations of valine, isoleucine, and leucine which, along with phenylalanine, adversely affected nitrogen retention when steers were fed the urea diets.

When comparing the urea purified diet with a natural diet a significant decrease in protozoal numbers with a significant

[2] Unpublished data, R. Orskov, 1966.
[3] Cline, T. R. 1965 Biological evaluation of purified diets for lambs with emphasis on the utilization of certain dietary components as affected by branched-chain volatile fatty acids. Dissert. Abstr., 26: 2399.

increase in bacterial numbers in the ruminal contents of steers was observed. Furthermore, during several sampling times, protozoa could not be detected in the ruminal contents when steers were fed the urea diet (22). Weller (23) reported that "comparing the essential amino acids of the bacteria with the protozoal hydrolysates the more important differences are the higher values with protozoa for isoleucine, leucine, phenylalanine, and in particular, lysine. These differences may help to account for the apparent nutritional superiority of the protozoal fraction of ruminal microbial proteins." In the present experiment plasma lysine concentrations were similar for both nitrogen sources. Additional information is needed to determine whether protein will support greater protozoal numbers when steers are fed purified diets and also to determine the amino acid requirements of protozoa and bacteria.

The concentrations of other free ninhydrin-positive compounds in the plasma are shown in table 5. The mean squares of ninhydrin-positive compounds showing significant differences are shown in table 4. Both hydroxyproline and glutamine plus asparagine were detected in greater concentrations ($P < 0.05$) in the plasma when the steers were fed the urea diets. Taurine and glutamine plus asparagine were detected in greater quantities ($P < 0.05$) with the starch-containing diets. Interactions ($P < 0.05$) were found for both hydroxyproline and citrulline with greatest concentrations found with the isolated soy-starch and urea-starch and glucose diets.

The nutritional significance of many of the other ninhydrin-positive compounds observed in the plasma is not readily apparent. However, taurine is needed for the synthesis of the bile acids, hydroxyproline is found in large quantities in collagen, glutamine and asparagine are both used in protein formation, whereas citrulline is used in the synthesis of urea in the mammalian body (24). Traces of phosphoserine, glycerophosphoethanolamine, phosphoethanolamine and 3-methylhistidine were detected in some of the blood samples.

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TABLE 5

Ninhydrin-positive compounds in the plasma of steers fed the experimental diets

Criteria [1]	Isolated soy		Urea	
	Glucose [2] + starch	Starch	Glucose + starch	Starch
	μmole/100 ml			
Taurine	0.6	0.8	0.6	1.2
Urea	184.3	232.4	136.4	373.2
Hydroxyproline	1.1	1.5	1.6	1.6
Asparagine and glutamine	10.6	11.7	12.2	17.1
Citrulline	4.8	6.5	5.8	4.6
α-Amino-*n*-butyric acid	0.5	0.5	0.7	0.5
Ornithine	5.8	5.4	4.2	6.0
Ammonia	16.6	16.9	15.1	17.1
1-Methylhistidine	0.4	0.3	0.5	0.4
Carnosine	0.8	0.8	0.7	0.9

[1] Each value is an average of 4 steers.
[2] Cerelose, Corn Products Company.

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Pantothenic Acid Deficiency in the Mink [1,2]

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ABSTRACT Ten female and 230 male kits were used to study the need for pantothenic acid by growing mink. Three trials were conducted using purified diets with ad libitum and equalized paired-feeding techniques. The minimal pantothenic requirement for growth was found to be 5 mg of calcium pantothenate/kg of diet, whereas 8 mg/kg of diet were required for optimal performance. Anorexia and reduced serum cholesterol were early responses to hypovitaminosis. The gross pathological findings were diarrhea, cachexia, and dehydration with hemorrhagic gastric ulcers and specific starvation lesions.

The need for pantothenic acid by most species is well documented. However, the requirements for the mink have not been investigated. The purpose of this study was to determine the pantothenic acid requirement of growing mink (*Mustela vison*), using purified diets.

MATERIALS AND METHODS

Experiment 1. One hundred male and 10 female kits, representing several color phases, were transferred from a standard ranch diet to a purified diet at 13 weeks of age. Five treatment groups received either a basal pantothenic acid deficient diet [7] or one of 4 levels of calcium pantothenate (4, 8, 12, and 15 mg/kg). The diets will be hereafter referred to as P0, P4, etc., denoting the level of calcium pantothenate per kilogram of diet. Twenty males were assigned at random to each of the 5 diets and the 10 females were fed the deficient diet. Details of the diets are included in tables 1, 2 and 3. The animals were weighed weekly and daily observations made as to their general welfare. Food was offered as required. When 50% of the animals fed the P0 diet had succumbed, three of the survivors in an advanced stage of hypovitaminosis were given oral doses of 2.5 mg calcium pantothenate daily for 3 days and offered the P15 diet.

Experiment 2. Ninety, pastel, mink kits were fed 5 experimental diets at 8 weeks of age, consisting of pantothenic acid-deficient diet (tables 1, 2 and 3) and 4 levels of calcium pantothenate (2, 5, 8 and 15 mg/kg of diet). Ten kits were assigned at random to the deficient diet and 20 each to the supplemented diets. The animals were weighed weekly and fed on alternate days, at which time individual feed intake data were taken. When 50% of the mink fed the P0 and P2 diets had died, the survivors were changed abruptly to the P15 diet and performance and food consumption data were recorded.

Experiment 3. Forty male mink kits of the dark color phase were used in an equalized paired-feeding study. A total of 5 kits was assigned at random to each of 4 diets (tables 1, 2 and 3) containing 2, 5 or 8 mg of calcium pantothenate/kg of diet. Each of the 20 kits was then pair-fed with a mink kit of similar body weight receiving a diet containing 15 mg of calcium pantothenate/kg of diet. The paired-feeding procedure was begun at 8 weeks of age. Individual daily feed intake and weekly weight records were recorded. After 28 days on experiment, 2 mink each from the P0 and P2 diet groups were killed along with their P15 pair-mates. The livers were

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[7] Containing a calculated level of 0.3 mg of pantothenic acid/kg of diet derived from vitamin-free casein (assay report from Mann Research Laboratories, New York).

TABLE 1

Composition of purified diets fed to growing mink

	Exp. 1	Exps. 2 and 3
Vitamin-free casein	30.00	30.00
L-Arginine·HCl	0.50	0.50
DL-Methionine	0.25	0.25
L-Cystine	—	0.25
Sucrose	39.00	39.00
Lard	10.00	10.00
Cottonseed oil [1]	9.00	9.00
Powdered cellulose [2]	5.00	5.00
Vitamin mixture	1.00 [3]	1.00 [3]
Mineral mixture	4.00 [4]	5.00 [5]
Choline dihydrogen citrate	—	0.66
Total	98.75	100.41
Choline chloride [6]	2 ml/kg diet	—
Ethoxyquin [7]	125 mg/kg diet	125 mg/kg diet

[1] Wesson Oil, The Wesson Oil Sales Company, Fullerton, California.
[2] Solka Floc (BW-40), Brown Company, Berlin, New Hampshire.
[3] See table 2.
[4] Phillips and Hart (7); obtained from General Biochemicals Inc., Chagrin Falls, Ohio.
[5] See table 3.
[6] Potency 70% in aqueous solution.
[7] Santoquin, Monsanto Company, St. Louis.

analyzed for dry matter and fat content using standard oven drying and ethyl-ether extraction techniques, respectively. Blood was drawn after 4 and 8 weeks on experiment by heart puncture and analyzed for serum cholesterol by the method of Schoenheimer and Sperry (8). The duration of this experiment was 8 weeks.

In all the experiments, assignment to diet was at random with the single restriction that dietary groups were balanced for body weight. The animals in all experiments were introduced to the purified diet during a 2-week period by a gradual dilution of the standard ranch diet.

RESULTS

Experiment 1. Anorexia was noted almost immediately in animals fed the deficient diet and the mink lost weight consistently after the first week. Deficient mink showed weight losses of 60 to 40 g/week for male and female kits, respectively (fig. 1). Fifty per cent of both sexes had died by day 57 of experimental feeding. The final body weight of the survivors at this stage was one-half of their original weight.

The deficiency of pantothenic acid was characterized only by nonspecific symp-

TABLE 2

Vitamin mixture used in mink purified diet (table 1)

	Exp. 1		Exps. 2 and 3	
	g/kg vitamin mix	*mg/kg diet* [1]	*g/kg vitamin mix*	*mg/kg diet* [1]
Thiamine·HCl	0.2	2	1.0	10
Pyridoxine·HCl	0.2	2	1.0	10
Riboflavin	0.4	4	2.0	20
Calcium D-pantothenate [2]	1.5	15	1.5	15
Niacin	4.0	40	4.0	40
i-Inositol	25.0	250	25.0	250
p-aminobenzoic acid	50.0	500	50.0	500
Menadione	0.5	5	2.5	25
L-Ascorbic acid	—	—	9.9	99
Folic acid	0.1	1	0.2	2
		μg/kg diet		*μg/kg diet*
Vitamin B_{12} [3]	4.0	40	4.0	40
Biotin	0.025	250	0.05	500
		IU/kg diet		*IU/kg diet*
dl-α-Tocopheryl acetate (275 IU/g)	14.54	400	14.54	40
Vitamin D_3 (3000 IU/g)	40.0	1,200	40.0	1,200
Vitamin A (250,000 IU/g)	4.8	12,000	4.8	12,000
Sucrose	854.735		839.510	

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

TABLE 3

Mineral mixture [1] used in mink purified diets (table 1)

	g/kg mineral mix
$CaCO_3$	81.01
$CaHPO_4 \cdot 2H_2O$	335.44
NaCl	133.02
$KHCO_3$	298.89
$NaIO_3$	0.126
$CuSO_4 \cdot 5H_2O$	1.58
$FeSO_4 \cdot 7H_2O$	15.03
$ZnSO_4 \cdot 7H_2O$	3.38
$ZnSO_4 \cdot H_2O$	0.10
$MgSO_4 \cdot 7H_2O$	127.48
$MnSO_4 \cdot H_2O$	3.52
Na_2SeO_3	0.011
$CoCl_2 \cdot 6H_2O$	0.162
$Na_2MoO_4 \cdot 2H_2O$	0.076

[1] Provided the following minerals to the completed ration at the following levels: (in per cent) Ca, 0.56; P, 0.53 (additional P in casein); Na, 0.26; K, 0.58; Cl, 0.40; I, 0.00041 (additional I in casein); Cu, 0.002; Fe, 0.015; Co, 0.0002; Zn, 0.004; Mn, 0.00609 (additional Mn in casein); Mg, 0.0625; S, 0.10097 (additional S in cellulose); and (in ppm) Se, 0.25 and Mo, 1.5.

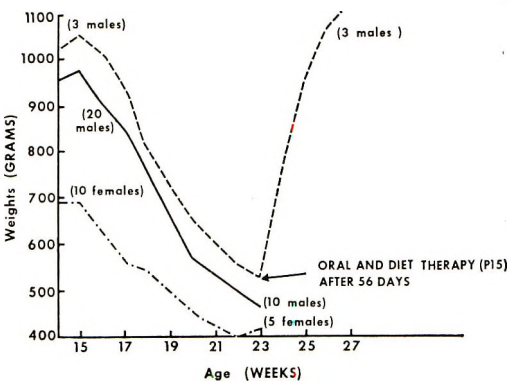

Fig. 1 Average weekly weights of male and female mink fed the deficient diet showing body weight losses and recovery of 3 male kits given vitamin therapy at 23 weeks of age or after 8 weeks of experimental feeding (exp. 1).

toms, as; 1) reduced feed intake with concomitant weight loss, 2) emaciation and dehydration, 3) vomiting following food or water intake during the later stages of hypovitaminosis, and 4) differences in fecal composition which can be described as a 2-phase process. First, the deficient animals demonstrated loose mucoid-like feces which started after they had been fed the deficient diet for approximately 21 days. This condition gave way to melena for the last 6 to 9 days prior to death. The gross pathology on the 36 mink examined was as follows: 1) starvation symptoms with the absence of fat in the coronary groove and kidney pelvis, 2) all but 2 mink dying from hypovitaminosis showed stomach ulceration and hemorrhage, 3) 4 animals had porphyrin-stained bile, and 4) petechial hemorrhages were observed throughout the intestine of 2 mink.

The 3 mink in an advanced stage of hypovitaminosis, which were given oral doses of calcium pantothenate and offered the P15 diet, showed a dramatic response in feed intake and weight gain (fig. 1). They attained the mean weight of the mink fed the P15 diet within 4 weeks. The incidence of diarrhea and melena disappeared completely on the first day of treatment.

Animals fed the P4, P8, P12 and P15 diets demonstrated consistent body weight increases with 2 exceptions (fig. 2). The mink fed the P15 diet showed a lower initial weight gain during the first week of the experiment. Between the twenty-third and twenty-seventh week of age the P4 group had an unaccountable loss and recovery of body weight. There were no significant differences at the twenty-third and twenty-eighth week of age in the average body weights of the groups fed supplementary pantothenic acid.

Blood glucose and plasma protein values showed no differences between normal and deficient animals sampled at 57 days of age. Analysis for liver fat from 8 mink at the terminal stages of hypovitaminosis yielded values which were at least one-half those of 4 animals fed the vitamin-supplemented diets (table 4).

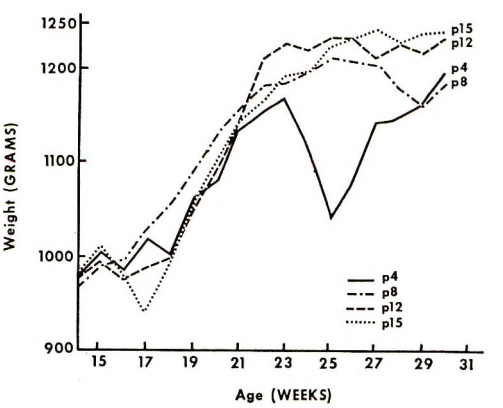

Fig. 2 Average weekly weights of mink fed 4, 8, 12 and 15 mg of calcium pantothenate (exp. 1).

TABLE 4

Liver fat composition, blood glucose and plasma protein values at 57 days of experimental feeding (exp. 1)

Diet	Sex	Liver	Blood	
			Protein	Glucose
		% ether extract [1]	*%*	*mg/100 ml*
P0 [2]	M	14.1	10.1	127
	M	7.0	10.4	95
	M	7.9	10.3	110
	M	14.5	11.0	86
	F	15.6	10.5	130
	F	9.0	10.2	114
	F	6.0	10.7	110
	F	6.1	10.7	84
P4	M	57.1	10.4	88
P8	M	41.0	10.5	117
P12	M	41.7	10.7	125
P15	M	31.5	10.8	130

[1] On dry-matter basis.
[2] Diet designated P0 = no calcium pantothenate supplement, P4 = 4 mg/kg, etc.

Experiment 2. Both the P0 and P2 diets resulted in a deficiency state, with 50% succumbing by 38 and 53 days, respectively. The deficiency symptoms were the same as those described in experiment 1. All deficient mink had persistent diarrhea which appeared at about day 18 in the P0 group and day 25 in the P2 groups. Persistent melena was observed in all but two of the animals in the former group at an average of 36 days. There was, however, no relationship between the incidence of melena and the time of death. Three animals fed the P2 diet demonstrated persistent melena. Mink fed the P5, P8 and P15 diets appeared to be normal in all respects.

The average food intake of mink in the P0 group decreased sharply during the ninth week of age and then reached a plateau at about 21 g/day (fig. 3). Weight losses averaged 70 to 80 g/week during the tenth and twelfth week of age. When 50% of the animals had died, the remaining 5 mink were given the P15 diet, which resulted in a positive response in feed consumption and body weight gain. The animals fed the P2 diet demonstrated an initial growth response followed by a similar pattern of performance as was obtained with the P0 group (fig. 4). A reduced feed intake was evident during the tenth week and weight losses commenced during the eleventh week. Food intake reached a plateau at about 30 g/day by the thirteenth week and did not vary appreciably during the next 2 weeks. The mink that survived for 53 days with the P2 diet were also given the P15 diet as therapy. The positive response to the supplement was striking.

No statistical differences were observed between the average weight gains in mink fed the P5, P8 and P15 diets (fig. 5). The P8 group had the lowest gain of the 3 groups over the 8 weeks of the experiment, whereas the highest gains were achieved with animals fed the P15 diet.

The average daily food consumptions for the P5, P8 and P15 groups are shown graphically in figure 6. Analysis of variance of weekly food consumption by each mink during the experiment gave highly

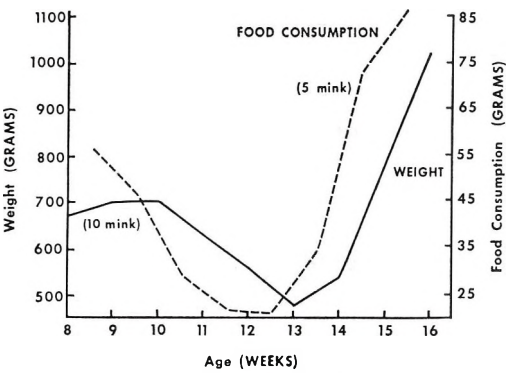

Fig. 3 Average weekly weights and average food consumption/mink/day for mink fed the basal deficient diet. Abrupt turn in curves follows the initiation of the diet containing 15 mg/kg of calcium pantothenate, 13 weeks of age (exp. 2).

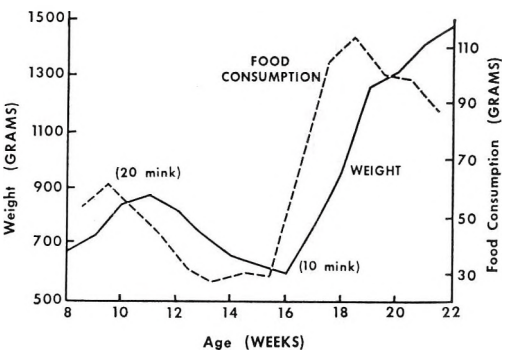

Fig. 4 Average weekly weights and average food consumption/mink/day for mink fed the 2-mg calcium pantothenate diet. Abrupt upward turn in the curves follows the initiation of the diet containing 15 mg/kg of calcium pantothenate at 16 weeks of age (exp. 2).

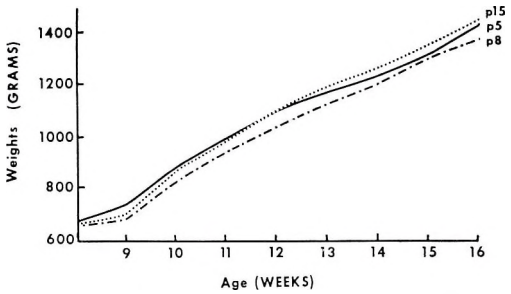

Fig. 5 Average weekly weights of mink fed the 5, 8 and 15 mg of calcium pantothenate/kg of diet (exp. 2).

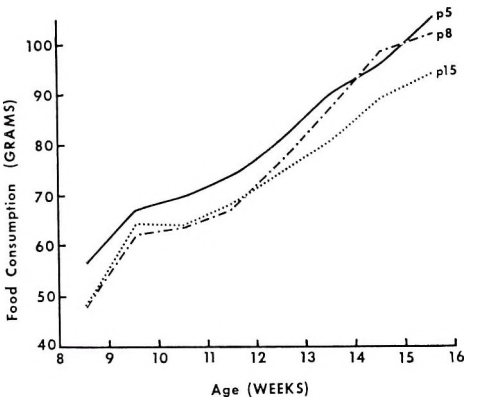

Fig. 6 Average food consumption/mink/day determined on a weekly basis for animals fed 5, 8 and 15 mg of calcium pantothenate/kg of diet (exp. 2).

significant differences ($P < 0.01$). Duncan's multiple range test (3) showed that the P5 group consumed significantly more food than the P15 group. Differences between other diets were not significant. There were no significant differences in the efficiency of food utilization, although the average values differed at different stages (fig. 7).

Experiment 3. In the equalized pair-feeding experiment, the dry matter determinations on the livers of animals destroyed after 28 days of feeding yielded similar values. Differences were obtained, however, with ether extract analysis (table 5). The P0 group had a higher liver fat content than their P15 pair-fed counterparts, whereas the P2 group showed the reverse situation. The relatively low liver lipid level of livers of mink fed P0 in experiment 1 (table 4) no doubt was due to the longer feeding period with a deficient diet. Serum cholesterol values for the P0 group, P2 and P5 diets in all cases were lower than the values obtained for their P15, pair-fed counterparts (table 6, parts *a* and *b*). To the authors' knowledge, there are no published blood cholesterol data for mink fed purified diets.

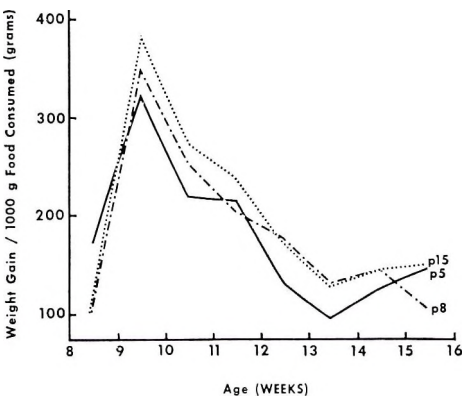

Fig. 7 Efficiency of feed utilization of mink fed 5, 8 or 15 mg calcium pantothenate/kg of diet (exp. 2).

TABLE 5

Liver dry matter and ether extract determinations of equalized pair-fed comparisons in experiment 3 *(28 days of feeding)*

Pair no.[1]	Diet	Dry matter	Ether extract [2]	Diet	Dry matter	Ether extract [2]
		%	%		%	%
1	P0 [3]	94	57	P15	95	40
2	P0	94	34	P15	94	25
3	P2	95	30	P15	95	38
4	P2	94	34	P15	95	43

[1] In each pair the P15 diet was equalized to the consumption of animal fed the lower supplemental or basal deficient diets.
[2] Ether extract percentage of dry matter.
[3] Diet designated P0 = no calcium pantothenate supplement, P2 = 2 mg/kg, etc.

TABLE 6

Serum cholesterol values of mink kits after 4 weeks and after 8 weeks of experimental equalized pair-feeding (exp. 3)

Pair no.[1]	Diet	Cholesterol	Diet	Cholesterol
		mg/100 ml		*mg/100 ml*
		Values after 4 weeks		
1	P0 [2]	5.5	P15	11.0
2	P0	10.5	P15	11.5
3	P2	7.5	P15	12.0
4	P2	7.5	P15	9.0
		Values after 8 weeks		
5	P2	3.0	P15	12.5
6	P2	4.5	P15	9.0
7	P5	8.0	P15	12.5
8	P5	12.0	P15	14.0
9	P8	13.0	P15	17.5
10	P8	14.0	P15	12.0

[1] In each pair the P15 diet was equalized to the consumption of animal fed the lower supplemental or basal deficient diets.
[2] Diet designated P0 = no calcium pantothenate supplement, P2 = 2 mg/kg, etc.

The food consumption, weight performance and feed efficiency of animals in experiment 3 were similar to those obtained in experiment 2. There were, however, no significant differences in feed utilization between inadequate vitamin-supplemented mink and their 15-mg pantothenate equalized pair-fed counterparts (table 7). In the P8 to P15 pair-fed comparison, the mink fed the P8 diet gained more rapidly ($P < 0.01$) and held a slight advantage in feed efficiency.

Sections of many animals were taken for histological examination and will be reported elsewhere.[8]

DISCUSSION

The essential nature of pantothenic acid in mink nutrition is clearly demonstrated for both male and female animals. The striking responses following vitamin therapy to animals in an advanced stage of hypovitaminosis, emphasize the specific requirement and action of pantothenic acid. Water intake was not estimated, but the mink made rapid recovery from dehydration as indicated by an apparent excess of 100% feed utilization during the first 5 to 7 days of vitamin therapy.

The calcium pantothenate supplementation of 4, 5, 8, 12 and 15 mg/kg of diet permitted maximal growth responses. The 5-mg level gave some indication of poorer diet utilization than higher levels, and the serum cholesterol values in this latter group were considerably lower than their pair-fed 15-mg diet counterparts.

From these results it has been concluded that mink introduced to a purified diet at 8 weeks of age require 5 mg/kg of diet of calcium pantothenate for maximal growth. This observation agrees with recently published work on the cat (4). However, at least 8 mg/kg of diet are required to obtain maximal feed efficiency and normal serum cholesterol values. This latter level is in keeping with the recommended requirements for the rat (1), mouse (6) and the chick (2).

Pantothenic acid requirement in the mink decreases with age as evidenced by a longer survival time when animals were

[8] McCarthy, B., L. Krook, H. F. Travis and R. G. Warner, in preparation.

TABLE 7

Performance of mink kits pair-fed purified diets containing several levels of pantothenate (exp. 3)

Paired groups	P0 [1]	P15	P2	P15	P5	P15	P8	P15
No. of pairs	4		3		5		5	
Days on test	26	26	47	47	54	54	54	54
Avg day's feed consumption, g	41	38	50	48	66	56	61	55
Total gain, g	−38	−33	210	202	543	528	545 **	414
Feed/gain	—	—	11	11	6.6	5.7	6.0	7.2

[1] Diet designated P0 = no calcium pantothenate supplement, P15 = 15 mg/kg, etc.
** $P < 0.01$.

introduced to a deficient dietary regimen at 13 weeks, instead of at 8 weeks of age. This has been reported for the dog and the rat (5, 9, 10).

There were no grossly visible hair changes nor signs of incoordination as experienced with other species. The latter symptom was difficult to evaluate since the mink at terminal stages of deficiency were very weak.

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Influence of Nutritional Deprivations in Early Life on Learning Behavior of Rats as Measured by Performance in a Water Maze [1]

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ABSTRACT Learning behavior was studied in rats that were subjected to different forms of nutritional deprivation in early life. Food deprivation during the first 3 weeks of life was achieved by increasing the number of rat pups nursing from one lactating female. At 3 weeks of age rats were weaned and some were fed an extremely low protein diet for 8 weeks. Four treatment groups were established by subjecting rats to either of these nutritional deprivations alone or by combining the 2 forms of restriction or by providing optimal nutrition from birth. When the rats were from 6 to 9 months of age, visual discrimination performance in a Y water maze was measured. Male rats that were deprived both before and after weaning made significantly more errors than the normal controls. The animals that were deprived pre-weaning or post-weaning alone gave intermediate results. No significant differences were obtained among female rats subjected to the same treatment regimens. The conclusion has been drawn that nutritional deprivation in early life can cause a long-lasting, possibly permanent retardation in the development of learning behavior. Motivational or emotional behavioral differences were noted among the treatment groups and therefore the relative contribution of "drive" as contrasted with "capacity" in the altered learning behavior is not known. When rats were tested for position reversal performance in the water maze shortly after weaning and during the time that certain groups were receiving the severely protein-deficient diet, the most errors were made by the double-deprived rats, followed closely by those that were malnourished only after weaning. Rats that had been restricted prior to weaning only or the normal controls made the fewest errors. Female rats showed a less definite effect of dietary treatment on performance than the males.

Ample evidence exists that the severely protein-calorie malnourished child exhibits behavioral abnormalities. Several groups that have submitted these subjects to psychomotor development tests, either during the acute phase of the deficiency or during various stages of recovery, have reported lower than normal test scores (1, 2). Low test scores appear to return toward normal within a 6-month recovery period, with improvement in scores being most rapid in those children who were the oldest at the time of hospitalization with severe malnutrition. Infants approximately 6 months of age when severe protein-calorie malnutrition developed, recovered satisfactorily during a 6-month period of hospitalization, but psychomotor test scores remained low (3). Studies such as these have led to the belief that severe malnutrition, at least of the type described as kwashiorkor, causes a retardation in mental development. Whether the behavioral abnormalities and the psychological test procedures have depicted a lowered "intelligence" or a retardation in the development of learning behavior has not been clearly established.

Numerous experimental animal studies have attempted to relate early food deprivation to subsequent adult behavior. Many of these have been reviewed and analyzed by Brozek and Vaes (4). In general, most of these studies have been difficult to interpret in terms of learning be-

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havior because food and water are the usual reinforcements used in the test procedures and prior food deprivations frequently cause strong motivational influences to be exerted which affect the performance of animals. In addition, food deprivations have usually been imposed after weaning and have been relatively mild in nature and for relatively short periods of time. Simulated protein-calorie malnutrition in which the kwashiorkor-like symptoms of fatty livers, extremely low serum proteins and gross edema has not been imposed on animals in which behavior studies were to be performed. In one series of studies rats were fed 2 levels of protein in "natural food" diets. In carrying the animals to the second generation and testing them in the Hebb-Williams maze test (5) evidence of lowered "intelligence" was noted in the male rats receiving the low protein diet although no significant change in maze test scores was obtained in the females (6). While many animal studies relating food deprivation to behavior have been conducted, little is known with respect to (a) the nutritional as compared with the psychological influences of the deprivation, (b) the component of behavior affected, that is, motivation or drive as compared with learning ability, (c) the permanence of altered behavior following nutritional rehabilitation, and (d) the relative influence of nutritional deprivations prior to weaning and immediately following weaning. The present study was designed to examine these factors.

EXPERIMENTAL

This research project was composed of 2 separate studies, one following the other, and each of approximately 10 months' duration. The basic experimental plan was the same for the 2 studies although some minor changes were made. The nutritional treatment of the animals was as follows: pregnant rats (Holtzman strain) were obtained so that all offspring that would be used were born during a span of 48 hours. By a system of random distribution the young were given to foster mothers for nursing, attention being given to avoid young being nursed by their own mothers. During the period of gestation and nursing, the females were fed the control high protein diet described in table 1. (In the first study a 4% protein diet was given from the nineteenth to the twenty-first day after birth.) Some foster mothers were given 16 young to nurse (in the first study 14 young/mother were used) and others were given 8 young to nurse. On day 21 the young were separated as to sex and distributed into groups that would receive either the high protein control diet or for the next 8 weeks a low protein diet (4% in study 1 and 3% in study 2). All rats were caged individually in a room maintained at 22° and provided feed and water ad libitum. At the end of the 8-week period the low protein groups were fed the high protein control diet. This control diet was continued for the remainder of the study. In this manner 4 dietary treatment groups of males and 4 identically treated groups of females were established, as illustrated in table 2.

In the first study, rats from 6 of the 8 groups were bled and serum proteins determined by the biuret method of Gornall et al. (7). Serum albumin was determined by paper electrophoresis. These measurements were made at the time the rats fed

TABLE 1

Composition of diets (in the second study the low protein diet contained 4.0% casein)

	Low protein	High protein
	%	%
Casein	4.7	25.0
Glucose monohydrate [1]	73.0	52.7
Hydrogenated vegetable oil [2]	15.0	15.0
Mineral salt mixture [3]	4.0	4.0
Choline dihydrogen citrate	0.3	0.3
B-vitamins in glucose monohydrate [4]	2.0	2.0
Fat-soluble vitamins in corn oil [5]	1.0	1.0
Total	100.0	100.0

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Primex, Procter and Gamble Company, Cincinnati.
[3] Mineral salt mixture: Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14*: 273, 1937; obtained from Nutritional Biochemicals Corporation, Cleveland.
[4] B-vitamins in 2.0 g glucose monohydrate in the following quantities: (in milligrams) thiamine·HCl, 0.40; riboflavin, 0.80; pyridoxamine·HCl, 0.40; Ca pantothenate, 4.00; niacin, 4.00; inositol, 20.00; biotin, 0.02; folic acid, 0.20; vitamin B_{12}, 0.03; menadione, 1.00.
[5] Fat-soluble vitamins in 1.0 g corn oil (Mazola, Corn Products Company) in the following quantities: (in milligrams) vitamin A acetate, 0.31; vitamin D (calciferol), 0.0045; α-tocopherol, 5.00.

TABLE 2

Experimental plan for nutritional deprivations during the pre-weaning (nursing) and 8-week post-weaning periods

Group no.		Experimental treatment	
Males	Females	Pre-weaning	Post-weaning
1	5	restricted	protein-deficient
2	6	restricted	normal
3	7	normal	protein-deficient
4	8	normal	normal

the low protein diet had reached their eighth week and were to be transferred to the normal control diet. When blood samples had been taken, the rats were killed and various soft tissues were fixed in Bouin's solution and bone and brain in 10% formalin. Demineralization was accomplished by 10% formic acid at pH 4.5 (buffered). Tissues were sectioned at 10 μ and stained with hematoxylin-eosin.

Behavioral testing was carried out primarily in a Y water maze which is illustrated in figure 1. Water at a depth which forced the rat to swim was maintained at 18°. A movable platform upon which the rat could climb and thus escape from the water was placed in one or the other of the 2 arms of the maze. The entire apparatus was kept in a darkened room. The first series of tests was conducted during the final weeks of the protein depletion period and consisted of a simple position reversal sequence which is described below. In the first study only the males were subjected to this testing, but in the second study both males and females were used. The position reversal test was divided into 3 stages, each stage consisting of 14 trials, with 2 trials being carried out each day. When both sexes were being tested, males were always tested first and females last, thus avoiding any effects of estrus perception by the males. In the first stage a platform was placed at the ends of each choice arm. The rats, as anticipated, established a position preference for either the right or left arm and would consistently go to that arm. In the second stage, the platform on the preferred side was removed so that the

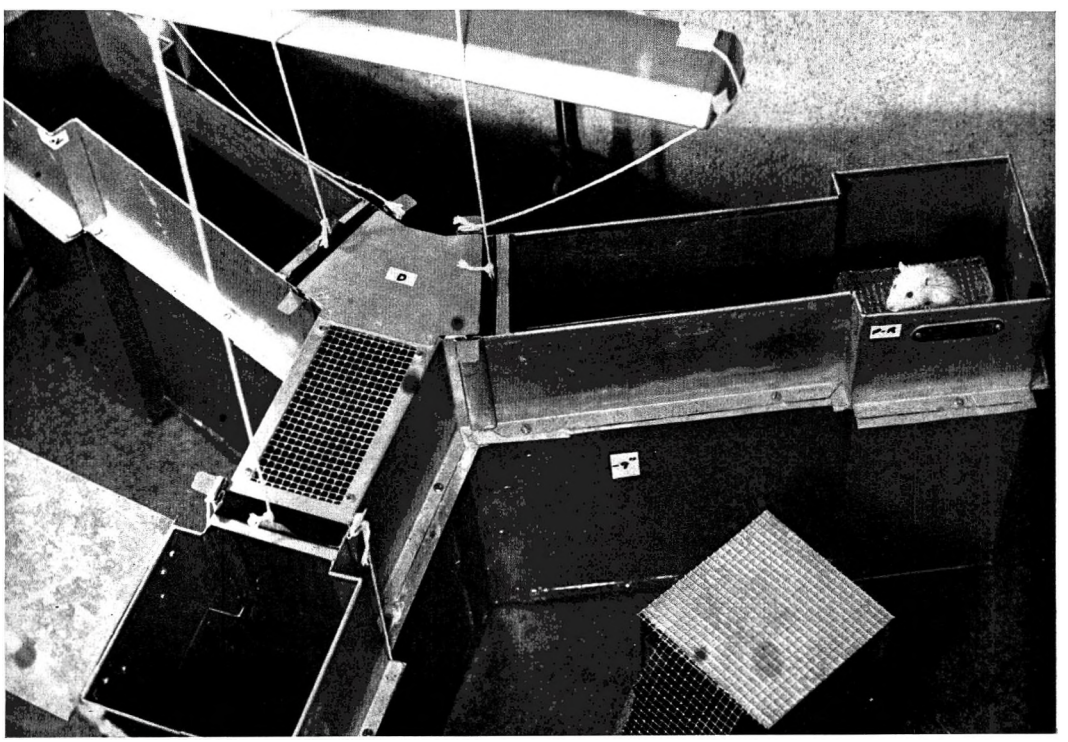

Fig. 1 Photograph of the Y water maze showing the escape platform and location of doors.

rat had to swim to the opposite arm. In the third stage, the rats were required to reverse back to their preference side by moving the platform back to the arm which they had originally preferred. Errors (a maximum of one per trial was recorded) were scored when the rat entered the arm that did not contain the platform. A record was also made of the elapsed time from opening the starting door until the rat had climbed up on the platform. Prior to starting the test all rats of one sex were placed for about 15 minutes in a special box which was connected to a portable hair dryer. When each rat finished its test it was dried with a turkish towel and returned to the warming box. When all rats of one sex had been tested, a repeat series was run so that each rat was tested twice. After the animals were thoroughly dry they were removed from the warming box and returned to the individual cages in which they were maintained. Females were always tested last and the same routine was followed that has just been described.

When the rats were approximately 6 months of age they were subjected to a visual discrimination test in the water maze. In the first study a black door and a white door were placed at the entrance of the 2 arms of the maze. The escape platform was always in the arm with the white door. The doors were pulled up so that they were about 1 cm above the surface of the water and their primary use as doors was when a rat entered the wrong arm of the maze the door could be lowered so the rat could not turn around and immediately swim to the other arm. The trapped rat that made an error was left in the water for a few seconds then lifted out and returned to the starting box so that the entire procedure could be repeated until it went directly to the arm with the escape platform. For purposes of evaluating performance, a maximum of one error per trial was credited to a rat no matter how many times that rat entered the wrong arm before locating the escape platform. A system of random numbers (8) was used to establish the sequential location of the white door and the associated escape platform. In the second study a 6-v light bulb was attached to a fixed partition in each of the arms of the maze. The light was switched on in the arm that contained the escape platform. Essentially the only difference in the second study was in the visual cue.

RESULTS

Growth curves for the males and females of the second study are shown in figures 2 and 3. In all essentials these results are confirmatory of the results in

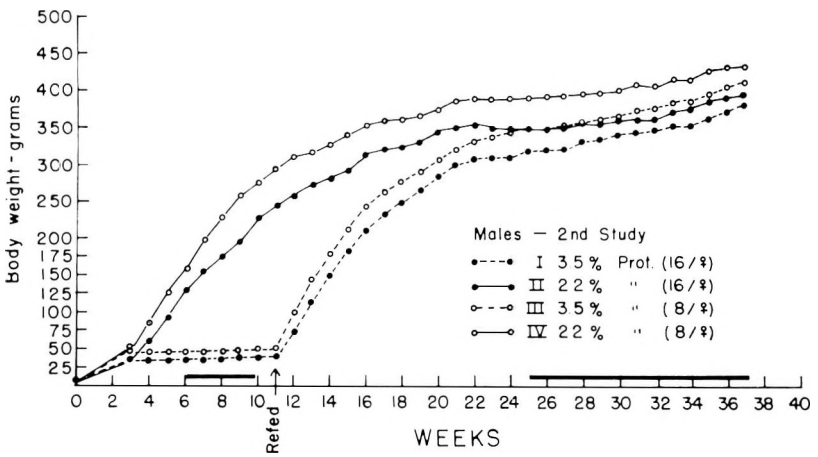

Fig. 2 Growth curve for males in the second study. The designations 16/♀ and 18/♀ indicate the number of pups nursed by one lactating female. The horizontal bars on the abscissa indicate the period during which behavioral testing was performed.

the first study, but with somewhat larger groups and consequently smoother curves, the second study was chosen for illustration. It is remarkable that the extent of stunting in the adult females (fig. 3) appears to be the same regardless of the type of restriction imposed early in life. Quantitatively the restriction post-weaning was much greater than the restriction pre-weaning, yet the ultimate body size after about 6 months' rehabilitation appears about the same. Perhaps even more remarkable is that the combination of pre- and post-weaning restriction appears to cause the same degree of stunting in the adult as either treatment alone. In the males (fig. 2) at 9 months the rats that were deprived only during the 8-week post-weaning period (group 3) had essentially caught up with the controls. Therefore, from this study it appears that in the males a pre-weaning deprivation of food resulted in a greater retardation of growth than post-weaning deprivation. A longer period of ad libitum feeding will be necessary to ascertain the conditions under which permanent stunting of growth is obtained.

Serum protein analyses are reported in table 3 for rats taken from the first study. The only changes were the highly significant decrease in both total protein and albumin in the groups that were fed the low protein diet post-weaning. Pathological examination at this time showed atrophy of tissues of rats fed the low protein diet. There were no specific lesions and the livers showed no evidence of fibrosis although there was some moderate fatty metamorphosis of liver cord cells, mainly in the periphery of the lobules in the post-weaning deprived rats. These animals also had markedly smaller corpuscles in the spleen. Brains showed no detectable changes histologically. The proximal tibial growth plate was the same

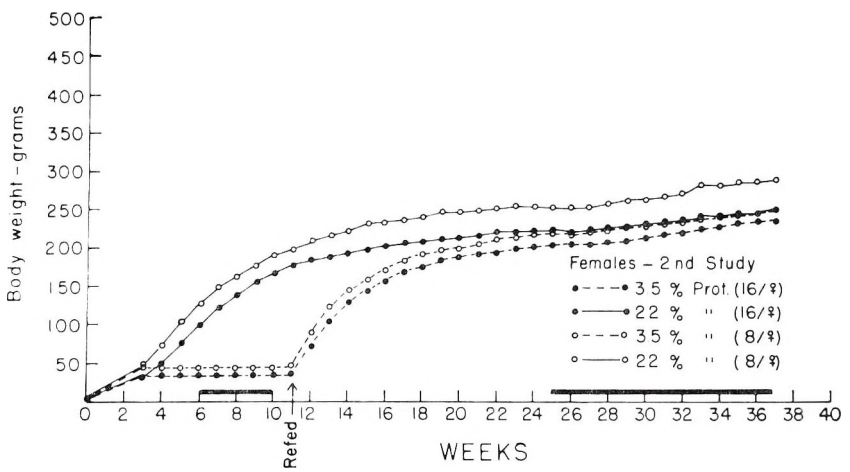

Fig. 3 Growth curve for females in the second study. The designations 16/♀ and 8/♀ indicate the number of pups nursed by one lactating female. The horizontal bars on the abscissa indicate the period during which behavioral testing was performed.

TABLE 3

Mean serum protein values for 6 groups of 3 rats each at 80 days of age taken from the first study [1]

	Group no.					
	1	2	5	6	7	8
Total protein	5.0 [a]	6.7 [b]	4.6 [a]	7.4 [b]	5.3 [a]	7.3 [b]
Albumin	2.4 [c]	4.2 [d]	2.5 [c]	4.3 [d]	2.9 [c]	4.4 [d]

[1] Means with superscripts not containing the same letter differ with a significance of $P < 0.01$.

width in all animals. In the post-weaning deficient rats there was no differentiation of cartilage cell layers although there was a marked decrease in basophilia of interstitial substance of the epiphyseal growth plate.

The results of the water maze position reversal test that was conducted during the immediate post-weaning period are given in table 4. In the first study only males were subjected to this test, but in the second study both males and females were tested. The male rats that were severely restricted post-weaning and were seriously deficient at the time of the tests (groups 1 and 3) made significantly more errors. The significance of differences in the female groups is not as clear-cut and it can only be concluded that the double-deprived animals in group 5 performed more poorly than the normal controls in group 8. As a generalization it seems logical to conclude that the male groups showed a more definite effect of deprivation than the females on this level of behavior. Furthermore, the immediate effects of protein deprivation were clearly evident, but there was some suggestion that the pre-weaning restrictions had an effect even though it could not be demonstrated by itself. Elapsed times to reach the escape platform for male rats in part C of the first study are shown in figure 4. The faster times of the 2 groups (1 and 3) that were deprived at the time of the test by post-weaning feeding of a 4% protein diet are clearly evident. This enhanced activity related to protein starvation has been noted by others (9, 10). It is interesting that the rats in these 2 groups reached the escape platform in shorter times, but made more errors in the process than was the case for the other 2 groups.

The visual discrimination testing was started when the rats were about 6 months old and rehabilitation had returned the groups to close similarity in total growth. The results of both the first and second study (table 5) indicate that with the males, the double-deprived animals of group 1 had significantly more errors than the normal controls of group 4. The significance of the relative positions of groups 2 and 3 remains in some doubt since these groups are reversed in the 2 studies. Nevertheless, in the second study where larger groups were used, group 2 which was deprived prior to weaning, made more errors than group 3, which was deprived after weaning. This is more clearly illustrated in the cumulative error curves of figure 5. The important point to be made is that deprivation prior to weaning and the deprivation after weaning each must have had an effect alone even though statistically the effect of the combined deprivation provides the most striking evidence of a long-term influence upon learning behavior. Furthermore, no significant difference existed between any of the groups

TABLE 4

Position reversal performance in the Y water maze during the immediate post-weaning period [1]

Study no.	Males				Females			
	Group no.				Group no.			
	1	2	3	4	5	6	7	8
	Stage A, mean no. trials to preferred side							
1	11.8(10) [2]	13.2(9)	12 (6)	12.8(6)				
2	11.1(14)	11.4(14)	11.3(15)	11.3(12)	11.3(12)	11.0(14)	12.1(9)	12.5(10)
	Stage B, mean errors swimming to side opposite preference							
1	3.0	2.3	2.0	2.7				
2	4.5^{a}_{x}	2.9^{ab}_{xy}	5.1^{a}_{x}	1.8^{b}_{y}	4.9^{a}_{x}	2.9^{a}_{xy}	4.7^{a}_{xy}	2.2^{b}_{y}
	Stage C, mean errors, position reversal							
1	3.4^{a}	1.2^{b}	1.8^{ab}	1.3^{b}				
2	4.9^{a}_{x}	2.9^{b}_{xy}	4.7^{a}_{x}	2.2^{b}_{y}	7.2^{a}	3.5^{ab}	3.8^{ab}	4.0^{b}

[1] Means with superscripts which do not contain the same letter have significant differences at $P < 0.05$ and similarly for subscripts the level of significance of differences is $P < 0.01$.
[2] Numbers in parentheses indicate number of rats/group.

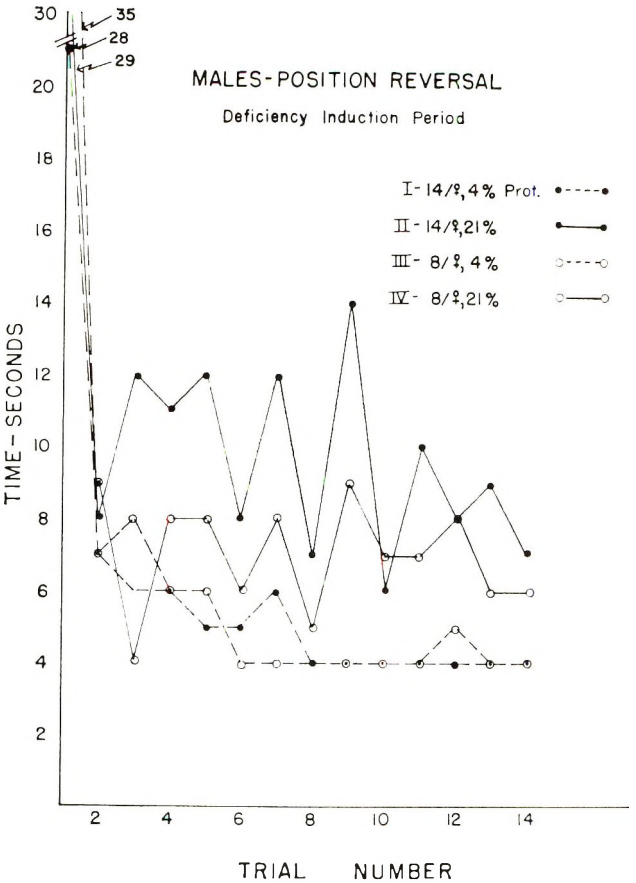

Fig. 4 Elapsed time to reach escape platform for male rats from the first study in the third stage of the position reversal test.

TABLE 5

Mean number of errors/rat in 50 trials in a Y water maze visual discrimination test [1]

Study no.	Males, group no.			
	1	2	3	4
1	22.5[a](9) [2]	20.0[ab](10)	20.8[ab](6)	18.3[b](6)
2	13.9[a](14)	13.2[a] (14)	11.7[ab](15)	10.1[b](10)
	Females, group no.			
	5	6	7	8
1	25.0[a](6)	23.7[a] (9)	20.8[a] (5)	25.0[a](5)
2	15.0[a](12)	12.0[a] (14)	15.0[a] (9)	14.4[a](9)
	Mean errors, all males		Mean errors, all females	
1	19.9[a]		23.7[b]	
2	12.4[a]		13.9[b]	

[1] Means with superscripts which do not contain the same letter have significant differences at $P < 0.05$.
[2] Numbers in parentheses indicate number of rats/group.

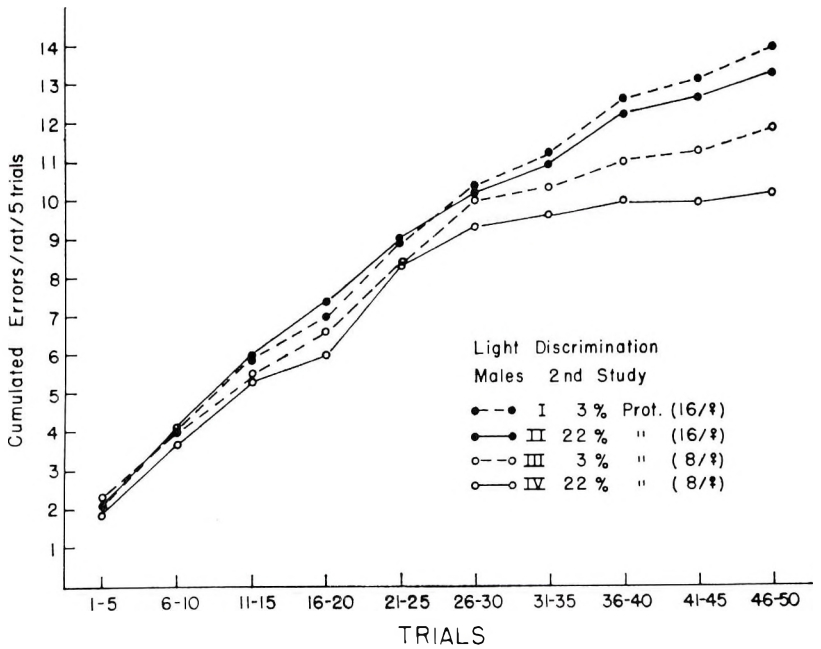

Fig. 5 Cumulative errors for male rats from the second study in the visual discrimination test.

of females in either study. Finally, at the $P < 0.05$ level of significance the females grouped together made more errors than the males in both studies.

DISCUSSION

The results that have been presented permit certain factual statements in that specific observations have been repeated in duplicate experiments. However, the interpretation of the meaning of the results will have to await considerably more depth in behavioral testing and experimental design for inducing nutritional deprivations. It has been found that a combination of pre- and post-weaning food deprivation in the male rats results in a higher than normal number of errors in a visual discrimination test. Single periods of deprivation either before or after weaning also appear to cause a poorer response, but the probability of these treatments being different is not as firmly established as the combination of the two. These changes in test results were observed about 6 months after the deprivations had been stopped and the rehabilitated rats were essentially the same in general appearance, although the early-deprived animals were somewhat smaller than the normal controls. This does not mean that the behavioral abnormality resulting from early deprivation was "permanent" although obviously the effect was long-lasting. In this series of visual discrimination tests no significant differences among the various treatment groups of female rats were observed in either the first or the second studies. Furthermore, in both studies when all 4 treatment groups were combined for each sex, a higher rate of errors was obtained in the females than in the males.

During the post-weaning period when a severe restriction in dietary protein was being imposed on certain groups, tests showed clearly that more errors were made in the groups that were severely malnourished by a combination of pre-weaning as well as post-weaning restriction (group 1 and group 5) as compared with normal controls (groups 4 and 8). This was true for the groups of male rats in both the first and second study and the

female rats in the second study. The male rats in the second study where larger groups were used also showed a significant increase in errors in the group that was solely deprived during the post-weaning period. These results suggest that pre-weaning undernutrition by itself was not sufficient to affect test scores in this simple position reversal test, but that the pre-weaning treatment did have a measurable additive effect upon post-weaning nutritional deprivation. It is not unexpected that the severely depleted rats with body weights only a fraction of the weights of those fed normally after weaning should show inferior performance in the maze test. These animals were severely depleted at the time the tests were run. With the same line of reasoning, perhaps it should not be unexpected that pre-weaning restrictions appeared to have an additive effect when combined with the severe protein depletion after weaning, since this combination depressed growth to a greater degree than either treatment alone. However, after 6 months' rehabilitation the combined restriction in the males (group 1) had a greater effect on test scores than either pre- or post-weaning restriction alone, particularly if results of both studies are examined. Using adult body weight as a criterion of the effect of earlier deprivation, all 3 experimental groups are essentially the same. In other words, here is an indication that in the rehabilitated rat, physical development and behavioral development do not necessarily parallel one another.

The fact that in the males of the second study the group that was restricted prior to weaning showed a significantly greater number of errors than the controls, whereas the post-weaning protein depletion did not, is interesting but is not considered as definitely established since this was not true in the first study. The more moderate interpretation is made that both forms of early deprivation probably had some long-term contributory influence upon behavior, but that other types of behavioral tests will be necessary to establish the validity of this conclusion.

Although the 2 studies reported here demonstrate conclusively that long-lasting behavioral abnormalities can be induced in rats by a combination of pre- and post-weaning nutritional deprivation, the interpretation and significance of these observations has not been established. Discrimination tests such as those used in the present studies have been considered a valid measure of learning behavior in animals. However, this form of behavior must be considered in terms of 2 components: first, the animal's motivation for performing a certain task, and second, its capacity for learning the task. Ideally, if an attempt is being made to measure learning ability, the factor of motivation must be ruled out or at least controlled in some manner. It was for this reason that a food reinforcement was not used for many earlier reports in the psychological literature illustrate the long-sustained food drives of rats that had been deprived of food in the early post-weaning period (11). An escape from cold water was assumed to be a form of reinforcement that would more likely affect all groups equally. Unfortunately, this proved not to be the case. In the long-term rehabilitated rats during the early stages of discrimination testing, it was evident that the normal control rats were more excited and more agitated in their attempts to get out of the maze than any of the experimental groups. They swam more rapidly to the escape platform. This was more noticeable in the males than the females and also more evident in the first than in the second study. However, even though based on subjective observation, there is no question that these are valid conclusions. There may have been other differences in degree of excitability among groups, but they were not sufficiently consistent to report. The excitability of the normal controls appeared to disappear as testing was continued. The relationship of the altered excitability of the rehabilitated experimental rats as compared with normal control rats, to their performance in the visual discrimination test cannot be assessed at this time. Therefore, the conclusion that early deprivation resulted in impaired learning behavior must include the reservation that it is not known how much of this impairment is due to altered motivation and how much to altered capacity. This difficulty is undoubtedly

common to most behavioral studies in which early food deprivation has been imposed, as is evident by the common conclusion that altered behavior in the adult animal is due to an effect of "drive" (11). To the authors' knowledge, in no study in which an early deprivation was followed by long-term rehabilitation has there been any evidence of an impaired learning capacity.

Motivational factors, at least as indicated by level of activity in the rats that were being studied in the water maze, were quite different during the acute phase of protein depletion as compared with the rehabilitated animals. During the postweaning period when the low protein diet was being fed, the animals were severely emaciated and their increased physical activity was to be expected since it is well-established that in animals, underfeeding increases physical activity. The very short lapsed time for the males in the 2 postweaning restricted groups (1 and 3) in the first study associated with the poorer performance scores for these 2 groups appears to separate motivation from learning performance. However, it is not unexpected that grossly stunted rats performed poorly. It does appear justifiable to propose that the poor maze scores reflected a learning defect that remained in evidence throughout a long-term rehabilitation period and was demonstrable 6 months later in poorer performance in a visual discrimination test. In a manner, this proposal tends to minimize the possibility that the higher performance scores of the controls were merely a reflection of their enhanced motivational drive. Further testing under a variety of experimental conditions that are devised so as to circumvent motivational influences interfering in the interpretation of results must be conducted.

A second limitation of the present study is in providing an understanding of the boundaries of nutritional deprivation that will produce long-lasting impairment in learning behavior. One major advancement has been the realization that the severe protein-calorie malnutrition associated with extremely low serum proteins, fatty livers, and gross edema such as found in the human infant condition of kwashiorkor is not essential for the development of long-term impaired learning behavior. Studies in the literature in which tests of learning behavior have been attempted following rehabilitation from some type of malnutrition are extremely scarce. In experimental phenylketonuria resulting from feeding high levels of phenylalanine to rats and mice, impaired learning ability has been demonstrated during the acute phase of malnutrition. Long-term impairment following rehabilitation has been observed only occasionally (12). Lát et al. (13) observed altered behavioral patterns in adult rats that had been underfed prior to weaning in a manner similar to that used in the present study. These behavioral abnormalities probably could not be interpreted as impaired learning behavior. Cowley and Griesel (6, 14) have been most successful in demonstrating impaired learning behavior due to the feeding of a somewhat protein-deficient diet. Although differences in the Hebb-Williams maze performance (5) were extremely small, large numbers of animals made these differences highly significant. It was suggested by these investigators that the poorer performance of the rats receiving the lower protein diet was due to the diet being fed not only through the period of gestation but also the period of nursing. However, the diet was not changed through 2 generations of rats so that there was no period of rehabilitation. If motivational factors are important in studies with animals that have been poorly nourished, then the experimental design used by Cowley and Griesel should have been particularly susceptible to this criticism. The test was conducted with rats that were being fed 2 levels of protein and food was used in the maze as a reinforcement. The diets were composed of natural foods and did not contain the same sources of protein. The gross protein content in one test was 20.1% for the high protein diet and 12.97% for the low protein diet. Interpolating from their published growth curves for the first filial generation a weight of about 275 g was reached at 300 days for the high protein group and 250 g for the low protein group. These weights are rather low and it is believed that these growth curves must have represented both males and females. The

fact that a 10% difference did exist in the adults is highly suggestive that this was due to effects of partial protein deprivation during gestation and lactation. However, the fact that no change was made in the diets makes it impossible to conclude that early deprivation had caused a permanent stunting of body size. There was a weight reduction in the parent rats that received the low protein diets only after reaching adulthood, which also confuses the interpretation of the cause of the 10% difference in the adults of the first filial generation. A very low level of probability separating the maze test scores of the 2 groups was observed in the first generation. Similar tests run on second generation rats receiving essentially the same diets gave highly significant differences in the Hebb-Williams test (6). Of particular interest in connection with the present study was that this difference in maze scores due to level of protein fed was significant only for the males. This very interesting sex difference in rats is strikingly similar to results obtained in the present study.

Aside from the observations reported here that show a long-lasting effect of 2 general forms of food deprivation, essentially nothing is known as to qualitative and quantitative components of the deprivation that are involved. More sensitive tests of learning behavior must be used and, since small differences are to be anticipated, it is probable that very large groups of animals must be used. One question of technique that must be considered is the relative contribution of psychological factors associated with the frustrations of young rats attempting to suckle in a situation where more pups than nipples exist. Is it justified to assume that behavioral responses that have been observed in the adult rat are solely the reflection of nutritional deprivation and have not been influenced by some other form of deprivation? The experimental psychologists who have studied the effect of litter size upon adult behavior relate their observations to "drive" or "stimulus" deprivation and possibilities of a "nutritional" deprivation are generally not considered (15, 16).

The final point for discussion is the possible relationship of present findings to the human being. The fact that behavioral abnormalities can be measured in infants with kwashiorkor and for a period of a few months following recovery appears to be well established (3). There is also evidence that in an economically depressed population, young children of the same age and socioeconomic background but different heights, psychomotor development in the taller children appears more advanced than in the shorter child (3). This supports the general concept that some measurements of mental development indicate a retardation due to early undernutrition that is not necessarily of the severe form of kwashiorkor. The interpretation of these psychological test procedures in terms of "intelligence" or learning behavior is difficult to establish, but presumably with additional studies, this type of information will become available.

On the other hand, the permanence of any behavioral change and the specific nutritional and critical age factors that may be involved will be established very slowly and much will depend upon information gained by animal experimentation. The practical importance of knowing more about nutritional effects upon mental development may lead to ill-advised conclusions in bridging the span from rat to man. In addition to the limitations of interpretation of animal studies that have been discussed above, it will be necessary to extend behavioral testing to a variety of animal species. Unpublished results from the laboratories of the authors have shown certain important differences in behavioral responses to early malnutrition between 3 species — rats, swine, and monkeys — that have been studied. The rat is useful in studies of this kind because of its rapid development. However, this very factor may be a serious handicap in transferring information from one species to another and particularly in extrapolating to man.

Despite all the limitations that have been mentioned, the results of the present study provide some basis for the belief that nutritional deprivations in early life may have a permanent retarding influence upon mental development. These results offer encouragement to continue and extend this area of experimentation.

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The Excretion Patterns of Vitamin B_6 and B_{12} in Preadolescent Girls [1]

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ABSTRACT During a 36-day study the excretion patterns of vitamin B_6 and B_{12} were investigated in 12 preadolescent girls. Diets composed of foods from plant sources provided 2 levels of protein, 22.1 and 40.3 g/day, and 2 levels of vitamin B_6, 1.30 and 1.73 mg/day, and were supplemented with vitamin B_{12} to give an intake of about 6 μg/day. The urinary excretion of both vitamin B_6 and 4-pyridoxic acid varied considerably from period to period for each subject. The average urinary excretion of vitamin B_6 was 1.1% of the lower intake and 0.79% of the higher intake, but the actual amounts were similar. The small excretion rates of both vitamin B_6 and pyridoxic acid may be indicative of intakes near the minimal requirement for these subjects. The average amounts of vitamin B_{12} excreted were 0.14 and 0.12 μg/day for the low and high protein diets, respectively, and represented 2.3 and 2.0% of the intake.

The present study, one of a series on preadolescent children, was undertaken to investigate the metabolic patterns of various nutrients in 7- to 9-year-old girls fed controlled diets devoid of animal products. This report presents data on the metabolism of vitamins B_6 and B_{12} in these children while eating diets providing 2 levels of protein from plant sources.

A recent review by Sauberlich (1) discussed the various methods used in estimating the vitamin B_6 requirements of humans and the limitations of present information. The need for additional data appeared to be particularly pertinent in the case of growing children for whom present recommendations are based largely on interpolations. Earlier studies of this series (2) provided information on vitamin B_{12} metabolism in preadolescent girls fed diets made up of foods from both plant and animal sources.

EXPERIMENTAL PROCEDURES

A detailed description of the study, information on the kinds and amounts of food in each of the diets, and data on the physical and biochemical status of each subject are presented in Southern Cooperative Series Bulletin no. 94 (3).

Twelve healthy girls ranging in age from 7 years and 10 months to 9 years and 5 months, in weight from 23.1 to 34.6 kg, and in height from 120.6 to 137.2 cm were subjects in a balance study consisting of 6 periods (periods 2–7) each 6 days in length and preceded by a 6-day adjustment (period 1). During the adjustment period all subjects were fed diet 13; they then were divided at random into 4 groups (diets 9, 10, 11 and 12) of 3 each. Subjects fed diets 9 and 10 consumed 22 g and those given diets 11 and 12, 40 g of plant protein daily during the 6 periods of the study. Diet 9 differed from diet 10 and diet 11 from diet 12 only in the supplemental nutrients supplied daily in capsules (3). Because the diets were completely devoid of animal foods, crystalline vitamin B_{12} was added to the bread to supply 6.0 μg per subject per day. Vitamin B_6 was supplied entirely by the foods.

Analyses were made on food and urine composites for each period. Total vitamin B_6 was determined on the daily food com-

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[1] This is paper no. XVIII in a series "Metabolic Patterns in Preadolescent Children." This study was part of the Southern Regional Research Project S-28, Requirements and Utilization of Selected Nutrients in Preadolescent Chilldren, supported in part by funds appropriated to the U.S. Department of Agriculture under the Research and Marketing Act of 1946 and the Hatch Act, as amended. The Human Nutrition Division of the Agricultural Research Service was a cooperator in this project.

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posites of periods 5 and 7 and on the urine composite of each subject for each period by the methods of Atkin et al. (4) and Parrish et al. (5) using *Saccharomyces carlsbergensis*. Pyridoxic acid was determined on urine composites by the method of Reddy et al. (6).

Vitamin B_{12} was determined on a composite of food for each period, and on the urine composite for each subject for periods 3, 5 and 7 using the *Lactobacillus leichmannii* AOAC assay method (7) as modified by Lichtenstein et al. (8).

RESULTS AND DISCUSSION

The average weights of subjects at the end of the adjustment period and the average daily intake of protein (N × 6.25), vitamin B_6 and vitamin B_{12} of subjects fed the 4 diets are summarized in table 1. Vitamin B_6 depended upon the types and amounts of foods in the daily menus. The vitamin B_6 content of daily food composites of the 2 periods used for analyses were similar but the range in daily amounts was considerable. The daily vitamin B_6 intakes of subjects eating the lower protein diets averaged 1.30 mg (1.14 to 1.62 mg), those of the children fed the higher protein diets averaged 1.73 mg (1.37 to 2.07 mg). The daily intakes of vitamin B_{12} were similar for the 4 diets; average intakes were around 6 μg/day in each period except in period 7 for diets 9 and 10 in which the intake decreased to 5.78 μg/day. As a result of this lower intake, the average intake for subjects fed diets 9 and 10 were slightly below the other 2 groups.

TABLE 1

Average weights of subjects and daily intakes of protein, vitamin B_6 and vitamin B_{12}

Diet no.	Avg wt of subjects	Average daily intake		
		Protein	Vitamin B_6	Vitamin B_{12}
	kg	*g*	*mg*	*μg*
9	26.3	22.1	1.30	5.98
10	27.4	22.1	1.30	5.98
11	30.0	40.3	1.73	6.06
12	27.8	40.3	1.73	6.06

The average vitamin B_6 intakes along with the urinary excretion of both vitamin B_6 and 4-pyridoxic acid are presented in table 2. The excretion of both vitamin B_6 and 4-pyridoxic acid varied considerably from period to period for each individual. There were no consistent changes in excretion patterns of either as the study progressed; the amount of either vitamin B_6

TABLE 2

Average vitamin B_6 intake, and urinary excretion of vitamin B_6 and 4-pyridoxic acid for each subject during six 6-day periods

Diet no.	Subject no.	Vitamin B_6 intake	Vitamin B_6 excreted		Pyridoxic acid excreted	
		μmole	*μmole/period*	*% intake*	*μmole/period*	*% intake*
9	45		0.50 ± 0.15 [1]	1.08	2.34 ± 0.90	5.05
	44	46.39	0.48 ± 0.16	1.03	2.65 ± 1.33	5.71
	50		0.41 ± 0.13	0.88	2.09 ± 0.71	4.51
	Mean		0.46 ± 0.14	1.00	2.36 ± 1.02	5.09
10	40		0.37 ± 0.02	0.80	1.87 ± 0.28	4.03
	46	46.39	0.66 ± 0.28	1.42	2.26 ± 0.82	4.87
	49		0.64 ± 0.34	1.38	1.64 ± 0.50	3.54
	Mean		0.56 ± 0.35	1.20	1.92 ± 0.58	4.15
11	41		0.50 ± 0.09	0.82	2.30 ± 1.04	3.79
	43	60.70	0.40 ± 0.11	0.66	2.00 ± 0.75	3.29
	47		0.54 ± 0.14	0.89	1.69 ± 0.67	2.78
	Mean		0.48 ± 0.11	0.79	2.00 ± 0.86	3.29
12	39		0.44 ± 0.12	0.72	2.62 ± 0.40	4.32
	45	60.70	0.47 ± 0.30	0.77	1.50 ± 0.38	2.47
	48		0.54 ± 0.30	0.89	2.21 ± 0.52	3.64
	Mean		0.48 ± 0.45	0.79	2.11 ± 0.63	3.48

[1] Mean ± SD.

or pyridoxic acid excreted in period 2 was not different from that in period 7. The average amounts of both vitamin B_6 and pyridoxic acid excreted did not differ among the 4 diets. However, the excretion of total vitamin B_6 and pyridoxic acid as a percentage of intake was greater in the 2 diets having the lower amount of protein and lower intake of vitamin B_6. The increased utilization of vitamin B_6 intakes which were excreted in the urine may indicate that the intakes were very near the actual requirements for these particular subjects. Excretion of 4-pyridoxic acid represented about 2.5 to 6% of the ingested vitamin B_6 on a molar basis (table 2) in contrast with approximately 50% reported by Reddy et al. (6).

The average daily urinary excretions of vitamin B_{12} for each subject during the study are presented in table 3. The amounts of vitamin B_{12} excreted ranged from 0.09 μg/day (subject 45) to 0.18 μg/day (subjects 46 and 41) and represented from 1.2 to 3.0% of intake. The amounts of vitamin B_{12} excreted were similar for all subjects and did not change appreciably after 36 days of the controlled diets. The intake of vitamin B_{12} during the adjustment period was 6.22 μg/day with an average excretion of 0.12 μg/day (range, 0.06 to 0.17), representing 2.0% of intake. In earlier studies (2) the excretion of vitamin B_{12} had decreased to a uniform level of around 0.30 μg/day when intakes had decreased to as low as 0.68 μg/day. The higher amounts of vitamin B_{12} being excreted in this study as compared with previous work appears to indicate intake above the minimum need for this vitamin. Subjects eating diet 12 had a somewhat lower excretion of vitamin B_{12} than other subjects during the study, but these 3 girls were at the lower end of the range of excretion during the adjustment period when all were receiving the same diet.

TABLE 3

Average daily urinary excretion of vitamin B_{12} for subjects maintained for 36 days with controlled diets

Diet no.	Subject no.	Average daily urinary excretion	
		μg/day	*% intake*
9	45	0.15	2.5
	44	0.14	2.3
	50	0.11	1.8
	Mean	0.13	2.2
10	40	0.11	1.9
	46	0.18	3.0
	49	0.14	2.4
	Mean	0.14	2.4
11	41	0.18	2.9
	43	0.13	2.1
	47	0.10	1.7
	Mean	0.14	2.2
12	39	0.11 [1]	1.8
	45	0.09	1.5
	48	0.10	1.7
	Mean	0.10	1.7

[1] Data only from periods 3 and 5. There was no measurable vitamin B_{12} in period 7 during which subject had received erythromycin propionate.

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Effect of Feeding Polyhydric Alcohols on Tissue Lipids and the Resistance of Rats to Extreme Cold

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ABSTRACT Three polyhydric alcohols, propylene glycol (PG), 1,3-butanediol (BD), and glycerol are being investigated as synthetic energy-yielding food compounds. These polyols were fed to growing rats within 2 environments in order to assess their relative calorie potential for growth, and to study comparative responses on epididymal adipose tissue and liver lipids. In addition, resistance to extreme cold (− 20°) was also measured. Weight gain was lower in rats fed BD or PG diets for 4 weeks, but was similar to that of controls when growth was calculated on a per unit food or calorie intake basis. Epididymal adipose tissue was lower in the groups fed BD and PG. Liver lipids and liver cholesterol increased in the rats fed PG. A lowered resistance to intense cold was exhibited in both BD and PG groups, which may indicate that the lowered fat stores of these animals were a causative factor in this response. Liver cholesterol was reduced by moderate cold exposure (5°) only in rats fed PG. The rats fed the glycerol diets did not show any of these metabolic changes observed in the groups fed BD or PG.

Compounds that can be chemically synthesized or derived from natural products, such as petroleum, and algae, offer a future potential as food sources. Our laboratories recently reported that the utilization of 1,3-butanediol, as a source of dietary energy in the rat, results in lowered epididymal adipose tissue lipids and a slight increase in liver and plasma neutral lipids (1, 2).

The purpose of the present paper is to describe the effects of feeding diets containing different non-toxic polyhydric alcohols on growth, liver lipids, liver cholesterol and epididymal adipose tissue of rats exposed to temperate (25°) or cold (5°) environments. In addition, resistance to intense cold (− 20°) was measured by comparing the survival rates of rats fed these compounds.

EXPERIMENTAL METHODS

Male weanling albino rats of 40 to 60 g each, of the CFE Carworth strain, were divided equally into the following 4 dietary treatments: 1) 30% fat (control); 2) 30% fat + 20% 1,3-butanediol (BD); 3) 30% fat + 20% propylene glycol; and 4) 30% fat + 20% glycerol. They were housed in individual cages and supplied with water and diets ad libitum.

These 4 diets were fed to: 1) 10 rats per each dietary treatment maintained for 4 weeks at 25°; 2) 10 rats per each dietary treatment maintained for 4 weeks at 5°; and 3) 18 rats per each dietary treatment maintained for 5 weeks at 25°, followed by exposure to − 20° until each group had 9 surviving rats (50% survival).

The composition of the 30% fat diet, and the 30% fat diet with 20% BD [2] substituted isocalorically for carbohydrates has been reported (1), except that agar and additional water (agar-gel-type diet) was not added to the diets in the present study. By rat-bioassay studies of Dymsza et al.[3] have shown that BD contains 5 to 6 kcal/g of net energy. Diets containing 20% propylene glycol and 20% glycerol had all other dietary ingredients identical with the BD diet. Propylene glycol has a gross energy value of 5.8 kcal/g (3). Hanzlik et al. (4, 5) have shown that increased growth was observed in rats fed

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[2] The authors wish to thank the Celanese Chemical Company, New York, for supplying the 1,3-butanediol.

[3] Dymsza, H. A., S. A. Miller and A. M. Browning 1963 Utilization of 1,3-butanediol as a synthetic source of dietary energy. Proc. 6th International Congress of Nutrition, E. and S. Livingstone, Lts., Edinburgh, p. 498 (abstract).

diets in which propylene glycol was substituted for dextrose on a weight basis, with equalized food intake to the control rats. It was estimated, therefore, that this glycol contains 4 to 5 kcal/g of metabolizable energy. The metabolizable energy supplied by glycerol was estimated as 4.1 kcal/g by Brambila and Hill (6) in the growing chicken, and this value was assumed in the present study.

After 4 weeks, 5 rats in each of the dietary treatments at 25° and 5° were killed. Liver was immediately excised, weighed, and total lipids extracted with chloroform-methanol (2:1). Non-lipid components were separated from the chloroform-methanol extract by means of partition chromatography on a Sephadex G-25 column as described by Therriault and Poe (7). Total lipid was determined gravimetrically after evaporation of the solvent under a stream of nitrogen at reduced pressure. Liver cholesterol was determined by a modified fluorometric procedure of Albers and Lowry (8). Both epididymal fat pads were quantitatively excised from each animal and weighed. The pads were dried in a vacuum oven at 50° to constant weight.

Statistical methods used in this study are described in Steel and Torrie (9). Regression analysis on the survival rates of the rats at − 20° was performed with the aid of a G.E. 225 Computer.

RESULTS AND DISCUSSION

The average weight gain, food intake, calculated metabolizable energy intake (ME), food and calorie efficiencies of rats fed the 4 diets for 4 weeks at 25° and 5° are presented in table 1. The food and calorie efficiencies of the rats consuming the diets with polyhydric alcohols are similar to the efficiencies of rats fed the 30% fat control diet at 25° or 5°. All animals maintained in the cold had food efficiency values about one-half that of rats in the temperate environment. This increased level of food consumption with smaller body weight gains reflects the increased calorie demands of the animal in cold acclimation (10). The calorie intake of the rats fed either the BD or propylene glycol diets was lower than that of the controls or glycerol-fed rats at 25°. However, at 5° the calorie intake of the rats fed propylene

TABLE 1

Average weight gain, food consumption, metabolizable energy intake (calculated), food and calorie efficiency of rats fed different polyols for 4 weeks at 2 environmental temperatures

	Control [1]		BD		PG		Gly	
	25°	5°	25°	5°	25°	5°	25°	5°
Weight gain, g	143 ± 10 [2]	116 ± 3	118 ± 3	81 ± 6	137 ± 6	102 ± 10	160 ± 5	116 ± 6
Food intake, g	319 ± 16	394 ± 9	257 ± 6	326 ± 7	290 ± 9	406 ± 15	342 ± 12	407 ± 12
Food efficiency [3]	45	29	46	25	47	25	47	28
ME [4] intake, kcal/rat	1627	2009	1285	1630	1392	1949	1607	1913
Calorie efficiency [5]	8.8	5.8	9.2	5.0	9.8	5.2	10.0	6.1

[1] Control indicates 30% fat diet; BD indicates 30% fat + 20% 1,3-butanediol diet; PG indicates 30% fat + 20% propylene glycol diet; Gly indicates 30% fat + 20% glycerol diet.
[2] Mean ± SE.
[3] As g gain/g food intake × 100.
[4] Metabolizable energy (calculated).
[5] As g gain/kcal intake × 100.

glycol was similar to that of the controls and of the animals fed glycerol. At 5° the BD-fed rats still had a relatively lowered calorie intake as compared with the other groups at this environment.

The epididymal adipose tissue weight, liver lipids, and liver cholesterol of rats maintained at the temperate and cold environments for 4 weeks are presented in table 2. In view of the excellent correlation between fresh weight of adipose tissue and extractable lipid content (11) it was felt that the epididymal adipose tissue weight could be used as a measure of alterations in adipose tissue lipids. The epididymal fat pads from rats maintained at 25° had a water content of 7 to 13%, whereas rats maintained at 5° had a water content of 10 to 23%. Boshart et al. (11) had observed that the water content of epididymal fat tissue increases with decreasing adipose tissue weight. In order to eliminate any variation due to water composition, we measured the dry weight of the epididymal fat pads. It was found that 93 to 97% of the dry weight was lipid consisting mainly of triglycerides.

All animals had reduced adipose tissue pads when exposed to the cold; however, rats fed the glycols, BD and propylene glycol, had significantly lower ($P < 0.05$) adipose tissue weight as compared with controls or glycerol-fed rats at both environments. In view of the lowered food intake in the rats fed BD and propylene glycol at 25° as compared with the control or glycerol-fed groups, the decreased epididymal adipose tissue weights may be merely a reflection of the lowered intake of calories. This does not appear to be the case since the calculated calorie efficiency (table 1) of all groups, within each of the 2 environments, was similar and the quantity of epididymal adipose tissue is presented in table 2 on a unit body weight basis. In addition, the calculated calorie consumption of the control, glycerol, and propylene glycol groups was similar in the 5° environment, yet the adipose tissue per 100 g body weight of the rats fed propylene glycol was still very much lower. This reduction in adipose tissue in the animals fed glycols may, therefore, indicate that there is an increased lipolysis of adipose tissue triglycerides with increased fatty acid mobilization, since previous investigations from these laboratories have shown that BD-fed rats have elevated plasma nonesterified fatty acids (2). Marsh and Whereat (12) observed that plasma nonesterified fatty acids represent the form in which fatty acids are mobilized from the fat depots.

Liver lipids and liver cholesterol were significantly higher ($P < 0.05$) in rats fed propylene glycol at 25° than in any of the other treatments. Cold reduced both the lipid and cholesterol content of the livers of rats fed propylene glycol. Treadwell et al. (13, 14) and Sellers and You (15, 16) observed that cold either decreases or prevents fatty liver in rats fed choline-deficient diets. Wood (17) has reported recently that cold had little or no effect on liver and other tissue cholesterol levels provided the diet was adequate in choline and inositol. In the present study, cold had no effect on the liver cholesterol levels of the controls, or the animals fed BD or glycerol.

Although the quantity of total liver lipids of rats fed BD at 25° was slightly higher than the liver lipids of the controls, analysis of variance showed no significant differences ($P > 0.05$) which is in agreement with our previous report (2). However, in the cold, the liver lipids of the BD-fed rats, as well as the propylene glycol group, was significantly increased ($P < 0.05$) as compared with the controls in the same environment.

When the rats were fed the 4 diets for 5 weeks and placed in the intense cold, the number of hours 50% of each group survived is illustrated by negative regression lines in figure 1. The rats consuming the glycols (BD or propylene glycol) withstood the cold only about one-half as long as the rats fed the control or glycerol diet. An analysis of variance of these data with a comparison of slopes (b values) of each line with the control group showed that the survival rate was significantly decreased ($P < 0.05$) when rats were fed the BD or propylene glycol diet. Feeding glycerol produced a survival rate similar to that of the controls. The amount of epididymal adipose tissue of rats at either 25° or 5° (table 2) appears to be correlated with the resistance of the animals to the ex-

TABLE 2

Epididymal adipose tissue weight, liver lipids, and liver cholesterol of rats fed different polyols for 4 weeks at 2 environmental temperatures

	Control [1]		BD		PG		Glycerol	
	25°	5°	25°	5°	25°	5°	25°	5°
Epididymal adipose tissue,[2] g/100 g body wt	1.27 ± 0.11 [a,3]	0.71 ± 0.06 [b]	0.84 ± 0.02 [b]	0.41 ± 0.04 [c]	0.89 ± 0.05 [b]	0.49 ± 0.04 [c]	1.38 ± 0.12 [a]	0.78 ± 0.05 [b]
Liver lipids, mg/g liver	68.19 ± 6.20 [bc]	52.01 ± 2.33 [d]	76.01 ± 5.21 [b]	68.43 ± 2.82 [bc]	99.50 ± 3.45 [a]	73.50 ± 3.41 [bc]	60.19 ± 5.36 [cd]	62.79 ± 2.00 [cd]
Liver cholesterol mg/g liver	2.38 ± 0.20 [cd]	2.28 ± 0.07 [cd]	2.84 ± 0.26 [c]	2.88 ± 0.08 [c]	6.63 ± 0.41 [a]	3.52 ± 0.20 [b]	2.42 ± 0.09 [cd]	2.03 ± 0.13 [d]

[1] Control indicates 30% fat diet; BD indicates 30% fat + 20% 1,3-butanediol diet; PG indicates 30% fat + 20% propylene glycol diet; glycerol indicates 30% fat + 20% glycerol diet.

[2] Dry weight.

[3] Mean ± SE. Means having a common letter in superscript are not significantly different ($P > 0.05$)

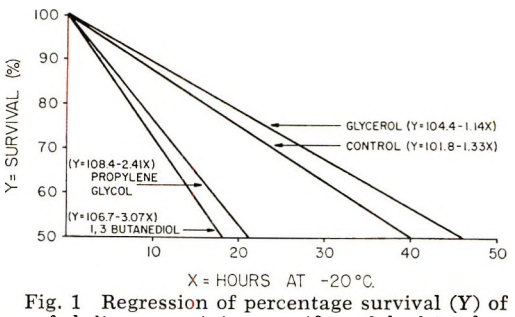

Fig. 1 Regression of percentage survival (Y) of rats fed diets containing specific polyhydric alcohols on number of hours (X) exposed to −20°. Diets were fed for 5 weeks prior to exposure.

treme cold. It is postulated that the quantity of epididymal adipose tissue is a good indication of the animal's resistance to intense cold. LeBlanc (18) observed that increased dietary lipids permitted higher sustained body temperatures of rats exposed to severe cold. Increases in dietary lipids have been shown in our laboratories to increase quantities of epididymal adipose tissue lipids in the rat (1,2).

Skin resistance and electromyogram measurements [4] were taken in rats fed the control 30% fat diet and BD at 25° and 5°. Normal increased skin resistance and shivering occurred in both groups of rats placed in the cold, indicating that rats consuming BD can initially respond to a lowered temperature.

From these studies it appears that feeding relatively large amounts of specific non-toxic glycols as a source of calories decreases the quantity of adipose tissue per gram of body weight which could be a causative factor in decreasing the resistance of the rat to intense cold. The mechanism whereby propylene glycol produces elevated liver lipids and liver cholesterol is unknown.

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[4] Grass Model 7 Polygraph, Grass Instruments, Quincy, Massachusetts.

Anti-thiamine Activity in Hawaii Fish [1,2]

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ABSTRACT Various species of fish found in Hawaiian waters were analyzed for anti-thiamine activity. Twenty-one of the 30 species tested contained thiaminase. A thermostable anti-thiamine factor was also usually present in the fish that contained enzymatic activity. Although anti-thiamine activity was more prevalent in certain families of fish, there was no constant correlation between taxonomic classification or the feeding habits or habitat and the presence of anti-thiamine activity.

The presence of thiaminase in fish has been known since Chastek paralysis, an acute dietary disease of foxes, was shown to be caused by the inclusion of raw carp in the diet (1). The nature and distribution of this enzyme has been reviewed by Yudkin (2) and Fujita (3). From past studies this enzyme appears to be more prevalent in fresh water fish than in ocean fish. Deutsch and Hasler (4) observed thiaminase in 15 of 30 fresh water species tested, whereas 9 species of salt water fish were negative. Marine fish which have this enzyme include garfish, the whiting and Pacific mackerel (5). Thiaminase has also been found in some Indian salt water and brackish water fish (6) and fish caught in the Gulf of Mexico (7). The presence of a thermostable anti-thiamine factor or factors in carp viscera has been reported by Somogyi and Kundig (8).

It was the purpose of the present investigation to analyze fish sold in Honolulu markets for enzymatic and thermostable anti-thiamine activity. Some fish products and other seafoods were also tested.

MATERIALS AND METHODS

Fresh fish were purchased in Honolulu markets and kept frozen until used. The anti-thiamine analyses were made within one week after purchase. Three or more determinations were made on each species. Fish tissue (10.5 g) was blended with 120 ml of phosphate buffer (0.5 M KH_2PO_4, pH 4.5) in a Waring Blendor for 5 minutes. The mixture was further homogenized in a Potter-Elvehjem homogenizer. One-half of the homogenate was boiled for 20 minutes in a hot water bath and served as the control. Twenty-milliliter samples of the boiled and unboiled homogenates, and 2 ml of thiamine solution (0.4 mg/ml) were added to 50 ml flasks and the pH adjusted to 4.5 if necessary. The flasks were placed in a Dubnoff shaking water bath at 38° for 4 hours and then boiled to stop enzyme action. Zero-time determinations were made on unincubated samples boiled immediately after the addition of thiamine. To facilitate precipitation of protein 3 to 4 drops of 2 N NaOH were added to each flask after it had been on the water bath for 15 minutes. Care was taken so that the pH did not exceed 6.0. The samples were cooled to room temperature and centrifuged. The supernatant was decanted and the precipitate washed with 10 ml of acidified H_2O, (pH 4.5), centrifuged and the supernatants were combined. This procedure recovered 99 to 100% of the added thiamine when no anti-thiamine activity was present. The supernatants were filtered through Whatman no. 1 filter paper and analyzed for thiamine by a modification of the method of Hochberg et al. (9).

RESULTS AND DISCUSSION

The anti-thiamine activity of the fish, fish products and other seafoods tested is shown in table 1. The presence of thiaminase in marine fish is more prevalent than

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[2] Published with the approval of the Director of the Hawaii Agricultural Experiment Station as Technical Paper no. 806.

TABLE 1
Anti-thiamine activity in Hawaii fish and seafoods

Scientific name	Hawaiian name	Common name	Anti-thiamine activity [1]	
			Enzymatic	Thermostable
Acanthurus dussumieri	palani	surgeonfish	0	0
Albula vulpes	oio	ladyfish	35	124
Aprion virescens	uku	green snapper	100	270
Caranx ignoblis or *C. sexfasciatus* (muscle)	papio	—	100	85
Caranx ignoblis or *C. sexfasciatus* (liver)	papio	—	518	211
Caranx mate	omaka	—	0	0
Chanos chanos	awa	milkfish	560	130
Coryphaena hippurus	mahimahi	dolphin	119	124
Decapterus pinnulatus	'opelu	mackeral (scad)	0	0
Etelis marshi	'ula 'ula	red snapper	118	101
Euthynnus yaito	kawa-kawa	little tuna	0	0
Katsuwonus pelamis (muscle)	aku	skipjack tuna	271	216
Katsuwonus pelamis (liver)	aku	skipjack tuna	1000	168
Kuhlia sandvicensis	aholehole	—	105	186
Makaira ampla or *M. audax* (white flesh)	a'u	marlin	0	0
Makaira ampla or *M. audax* (orange flesh)	a'u	marlin	77	111
Family Moridae	—	black cod	124	230
Mugil cephalus	'ama 'ama	gray mullet	70	114
Mulloidichthys auriflamma	weke'ula	goatfish	106	44
Mulloidichthys samoensis	weke 'a 'a	goatfish	249	432
Myripristis berndti	u'u	squirrelfish	0	0
Neothunnus macropterus	ahi	yellowfin tuna	265	95
Parupeneus multifasciatus	moano	goatfish	128	0
Polydactylus sexfilis	moi	threadfish	110	92
Priacanthus cruentatus	'aweoweo	—	0	0
Pristipomoides microlepis	'opakapaka	red snapper	265	35
Scarus dubius	uhu	parrotfish	30	79
Scomberoides sancti-petri	lae	leatherback	46	76
Seriola dumerilii	kahala	amberpack	0	0
Sphyraena barracuda	kaku	barracuda	0	111
Trachurops crumenophthalmus	akule	bigeye scad	184	167
Xiphius gladius	a'u	broadbill swordfish	155	28
		Prepared fish		
Katsuwonus pelamis	aku	tuna, freeze dried	970 [2]	1290 [2]
Katsuwonus pelamis	aku	tuna, salted dried strips	564 [2]	212 [2]
—	—	tuna, canned Haw'n branch	0	0
—	—	Herring, dried salted	485 [2]	277 [2]
—	—	salmon, salted	93	215
		Other seafoods		
Ommastrephes hawaiiensis	—	squid (flesh)	254	61
Helcioniscus sp.	opihi	Hawaiian limpet	75	91
Polypus marmoratus	—	octopus (cooked)	0	0
—	—	shrimp	0	0
—	—	clam	2640	120

[1] Anti-thiamine activity is expressed as micrograms of thiamine destroyed/100 g of fish tissue (wet weight) per hour.
[2] Activity is expressed as dry weight.

had been indicated by previous studies. This enzyme was present in 21 of the 30 species studied, in squid and limpet and also in the dried fish products. The thiaminase levels are similar to those reported by Fujita (3). The liver of the aku and the papio had 3 to 4 times the activity of the muscle. Other organs such as intestines, kidney and liver generally showed higher activity than muscle (3).

It became evident in our first studies that all of the anti-thiamine activity could not be destroyed by heating and that a thermostable thiamine-destroying factor was present. Thermostable anti-thiamine activity has not been reported previously in fish muscle. The thermostable factor was generally present in all species containing enzymatic activity. However, the moano (goat fish) had enzyme activity but no thermostable activity, whereas the kaku (barracuda) had thermostable activity but no enzyme activity. Both of these fish would be considered to have a low or trace amount of activity. A thermostable anti-thiamine factor crystalized from carp viscera by Somogyi and Kundig (8) was reported to be a large molecule containing amino acids but not a typical protein. The fish muscle thermostable factor may be a similar substance.

Although the presence of thiamine-destroying activity appears to be more prevalent in certain families there is no consistent correlation. Of the 3 species of tuna tested, 2 contained activity, whereas one did not. Activity was observed in 3 species of the snapper family and 3 species of the goatfish family. The diet and habitat of the fish might be factors in determining whether a fish possesses thiamine-destroying activity. However, the information available on feeding habits and habitat (10) offers no basis for prediction as to whether a fish possesses thiaminase activity, thermostable activity, or both.

The biological significance of the ingestion of fish containing anti-thiamine activity is unclear. The work of Melnick et al. (11) indicates that considerable destruction of thiamine in the gastrointestinal tract of man can take place when clams, which contain thiaminase, are eaten. Fish are frequently eaten raw in Hawaii as "sashimi." The most popular species used in this way are aku and ahi, both of which contain thiaminase activity. It is a possibility that the destruction of dietary thiamine could result from eating fish containing anti-thiamine activity if consumed in sufficient quantity. The presence of anti-thiamine activity should be considered in the use of fish flours for high protein food supplements for human use and also in the use of fish products in animal feeds.

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Effects of Biotin on Folic Acid Metabolism in the Rat

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ABSTRACT Interrelationships between biotin and folic acid observed previously are confirmed in this paper. Data obtained indicate that in biotin-deficient rats the liver storage of most of the various folate derivatives was significantly lower than that in control rats, whereas in folic acid- and biotin-deficient rats the quantities of these compounds were lower than those in folic acid-deficient rats. In doubly deficient rats the amount of formiminoglutamic acid excreted in the urine was markedly higher, when compared not only with that of control rats but also of folic acid-deficient rats. After injection of folic acid the biotin-deficient rats excreted smaller quantities of folate metabolites and showed lower liver levels of various folate derivatives than those of control rats. In doubly deficient rats greater alterations of the urinary excretion of folate metabolites and of liver storage of various folate derivatives were observed as compared with the rats deficient only in folic acid. The liver homogenates of biotin-deficient rats converted folic acid to citrovorum factor less effectively than those of control rats, whereas liver homogenates of doubly deficient rats showed less capacity for conversion when compared with the folic acid-deficient rats. Therefore the results demonstrate the influence of biotin in folic acid metabolism and in particular in its utilization for the biosynthesis of coenzymatic derivatives.

Relationships between biotin and folic acid have been studied both in microorganisms and in mammals. Mitbander and Sreenivasan (1) observed that biotin causes a marked stimulation of folic acid biosynthesis by growing cells of *Lactobacillus arabinosus* 17–5. More recently Dias et al. (2) showed that folic acid could replace biotin for the growth of some species of *Corynebacterium barkeri*.

In mammals the relationship between the 2 vitamins was first observed by Luckey et al. (3). These authors, during a vitamin balance study with germfree rats, noted that the administration of biotin caused a marked increase in folic acid excretion. This result was interpreted as tissue synthesis of folic acid dependent upon biotin.

According to Noronha et al. (4) biotin may influence the synthesis of folic acid by intestinal microflora. This hypothesis was suggested since the administration of biotin to rats deficient in both biotin and folic acid caused, in addition to an almost complete restoration of liver levels of folic acid, an abnormal increase in folic acid excretion in feces but not, however, in urine. Since the biotin-treated animals were deficient in folic acid, it is unlikely that the large amounts of folic acid excreted in feces were of tissue origin; it is more probable that they were caused by increased synthesis due to intestinal microflora.

More recently Marchetti et al. (5, 6) demonstrated that biotin, in addition to stimulating intestinal synthesis of folic acid, appears also to be able to influence the enzymatic process of conversion of folic acid to its coenzymatic forms. In fact it has been observed that the administration of biotin induces a significant increase in the content of various folate derivatives in the liver of biotin-deficient rats and in the amount of citrovorum factor (CF) formed in vitro by liver homogenate.

The present research was undertaken to study the role of biotin in folic acid metabolism. For this purpose biotin and folic acid were examined comparatively for their effect on 1) the content of folic acid-active substances and their distribution in the liver, 2) the conversion of folic acid to activated forms in vitro by liver homogenate and in vivo by intact animals, and 3) the urinary excretion of formiminoglutamic acid in biotin and folic acid-deficient rats.

EXPERIMENTAL

Animals and diets. Weanling male rats of the Wistar strain, weighing 45 to 50 g,

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were used for all experiments. The animals were divided into 4 groups of 50 rats each. They were housed in wire-bottom cages and each group was fed ad libitum one of the following diets: 1) purified basal diet, 2) biotin-free diet, 3) folic acid-free diet, and 4) biotin- and folic acid-free diet. The composition of these diets is given in table 1. After 60 days the animals of the 4 groups were used for the various determinations.

Total content and distribution of the various folate derivatives in the liver. Six animals of each group were killed and the livers were homogenized with 2 volumes of water, and then 2 volumes of ice-cold acetone were added. The precipitated materials were washed with acetone and ether and dried over P_2O_5. For determining the concentration and distribution of folate derivatives, acetone powders were extracted in 0.1% (w/v) potassium ascor-

TABLE 1

Percentage composition of experimental diets

	Group 1	Group 2	Group 3	Group 4
	%	%	%	%
Casein, vitamin-free	8	8	8	8
Autoclaved egg white [1]	10	—	10	—
Raw dried egg white	—	10	—	10
Starch	59.5	59.5	59.5	59.5
Sucrose	10	10	10	10
Salt mixture no. IV [2]	4	4	4	4
Peanut oil [3]	4	4	4	4
Shark oil	2	2	2	2
Succinyl sulphathiazole	2	2	2	2
Vitamin mixture [4]	0.5	0.5	0.5	0.5
Biotin	0.0001	—	0.0001	—
Folic acid	0.0003	0.0003	—	—

[1] Autoclaved at 120° for 30 minutes and dried.
[2] Hegsted et al. (13).
[3] Fortified with α-tocopherol (250 mg/liter).
[4] The vitamin mixture contained (mg/g): thiamine·HCl, 0.8; riboflavin, 1; pyridoxine, 1; niacin, 2; Ca pantothenate, 4; menadione, 0.6; inositol, 100; choline·HCl, 100.

TABLE 2

Effect of biotin and folic acid on body weight gain of biotin- and folic acid-deficient rats

Group [1]	Treatment	Initial wt	Final wt	Wt gain
		g	g	g
1	Control	53 ± 0.93 [2]	232 ± 6.25	179 ± 7.31
2	Biotin-deficient	52 ± 0.87	150 ± 5.49	98 ± 6.02
3	Folic acid-deficient	55 ± 1.10	145 ± 8.75	90 ± 9.13
4	Biotin- and folic acid-deficient	56 ± 1.07	95 ± 7.85	39 ± 8.79

[1] Group 1, basal diet containing 10% autoclaved egg white + biotin (1000 μg/kg) + folic acid (3000 μg/kg); group 2, basal diet containing 10% raw dried egg white + folic acid (3000 μg/kg); group 3, basal diet containing 10% autoclaved egg white + biotin (1000 μg/kg); group 4, basal diet containing 10% raw dried egg white.
[2] Each value is given as the mean ± SE of mean of 50 animals.

TABLE 3

Effects of biotin and folic acid on folate activities in the liver of rats

Group	Treatment	Folic acid activity assays		
		P. cerevisiae	*L. casei*	*S. faecalis*
		mμg/g fresh tissue	*mμg/g fresh tissue*	*mμg/g fresh tissue*
1	Control	1,134 ± 99.5 [1]	4,042 ± 345.5	1,676 ± 101.2
2	Biotin-deficient	851 ± 69.7	2,664 ± 311.6	1,408 ± 61.8
3	Folic acid-deficient	206 ± 30.5	640 ± 51.2	389 ± 31.4
4	Biotin- and folic acid-deficient	107 ± 12.2	501 ± 40.3	204 ± 19.3

[1] Each value is given as the mean ± SE of mean of 6 determinations on different animals.

bate, pH 6.0, by heating the suspension (20 mg/ml) for 30 minutes in a water bath at 75°. The extract was chilled at 0° and then centrifuged. Aliquots of clear supernatant were put on a column of DEAE-cellulose-Hyflo Supercel mixture (1:1,25 w/w) prepared for use by washing first with 0.5 N KOH, with water until the washing was neutral, with 0.5 M potassium phosphate buffer, pH 6.0, and finally with water until the washing was free of phosphate. After the extract was absorbed the column was washed with small portions of 0.2% potassium ascorbate solution. Then 40 ml of 2% ascorbate solution were placed on the column and 0.5 M potassium phosphate buffer, pH 6.0, containing 0.2% ascorbate was put on a separating funnel attached to the column and was passed drop-by-drop through the head of the ascorbate solution. The flow rate was 1 ml/minute. Fractions of 5 ml were collected in tubes containing 0.1 ml of 10% potassium ascorbate, pH 6.0, and stored at 0° until the assay was made.

All the eluted fractions, after suitable dilution in 1% potassium ascorbate, pH 6.0, were analyzed for folic acid growth activities with *Pediococcus cerevisiae* ATCC 8081 (*Leuconostoc citrovorum*), *Streptococcus faecalis* R ATCC 8043 and *Lactobacillus casei* ATCC 7469. The media used were those described by Bakerman (7), calcium leucovorin being used as a reference standard. The components of folate activities were differentiated from each other by using the criteria described previously (8).

Conversion in vivo of folic acid to activated forms. The efficiency of converting folic acid in its activated forms was studied. After injection of folic acid, determinations were made of either the folate metabolites which appear in urine, or the total content and distribution of active folate derivatives in the liver. Eight rats of each group were injected intraperitoneally with 200 µg folic acid/100 g of body weight. The rats were placed in individual metabolism cages and urine samples were collected for 12 hours in bottles containing potassium ascorbate (100 mg). The citrovorum factor (CF) activities in urine were assayed microbiologically with *P. cerevisiae*. The assay was carried out

TABLE 4

Effects of biotin and folic acid on the distribution of various folate derivatives in the liver of rats

Compounds	Liver concentration of folate derivatives			
	Control	Biotin-deficient	Folic acid-deficient	Biotin- and folic acid-deficient
	mµg/g tissue	*mµg/g tissue*	*mµg/g tissue*	*mµg/g tissue*
N^{10}-Formyltetrahydrofolic acid	859 ± 72.50 [1]	610 ± 51.30	123 ± 20.60	74 ± 10.50
N^{10}-Formyltetrahydrofolic acid and N^{10}-formylfolic acid	1,074 ± 99.22	905 ± 79.45	103 ± 17.85	72 ± 8.25
N^{5}-Formyltetrahydrofolic acid	160 ± 14.61	110 ± 9.98	41 ± 0.75	19 ± 2.55
N^{10}-Formyltetrahydropteroyltriglutamic acid	1,084 ± 119.53	566 ± 10.60	173 ± 21.05	180 ± 17.20
Tetrahydrofolic acid	189 ± 21.00	224 ± 31.69	66 ± 0.71	13 ± 2.06
N^{10}-Formyl derivatives of a pteroylpolyglutamic acid	415 ± 39.55	125 ± 12.27	35 ± 0.25	30 ± 2.75
N^{5}-Formyltetralhydropteroyltriglutamic acid	220 ± 28.27	55 ± 36.39	50 ± 1.05	11 ± 1.98
N^{5}-Formyl derivatives of a pteroylpolyglutamic acid	110 ± 15.30	59 ± 47.20	50 ± 0.87	11 ± 2.50

[1] Each value is given as the mean ± SE of mean of 6 determinations on different animals.

on urine samples diluted in water ("CF-regular" activity), on urine samples autoclaved at 114° for 30 minutes with potassium ascorbate, pH 6.2 ("CF-autoclaved" activity) and on urine samples diluted in 0.6% potassium ascorbate and steamed for 30 minutes ("CF aseptic" activity). Immediately, after collecting the urine, the rats were killed and the livers were used for determining total content and distribution of folate derivatives according to procedures described above.

Conversion in vitro of folic acid to citrovorum factor. Six animals of each group were killed and the livers were homogenized with 4 volumes of 0.08 M phosphate buffer pH 6.3. Aliquots of homogenates were incubated in a reciprocating shaker at 37° for 2 hours under nitrogen with 10 mg L-serine, 10 mg DL-homocysteine and 100 μg of folic acid.

The amounts of CF formed in the systems were assayed microbiologically with *P. cerevisiae*.

Urinary excretion of formiminoglutamic acid. For measuring formiminoglutamic acid (FIGlu) in urine, 8 rats of each group received 1 ml of 0.005 M L-histidine by stomach tube and 5 ml of 0.15 M NaCl by intraperitoneal injection. The rats were placed in individual metabolism cages and urine samples were then collected for 7 hours. Formiminoglutamic acid was determined by measuring spectrophotometrically at 365 mμ the N^5,N^{10}-methenyltetrahydrofolic acid formed by incubating at 25° for 30 minutes aliquots of urine with suitable amounts of FIGlu-transferase, formiminotetrahydrofolic acid-cyclodeaminase, DL-tetrahydrofolic acid, and 2-mercaptoethanol (9). All the results were analyzed for statistical significance by Fisher's *t* test; a difference between 2 means was regarded as significant when *P* was not greater than 0.05.

RESULTS

After 60 days the animals receiving the biotin- and folic acid-free diet showed more retarded growth and more severe symptoms of deficiency as compared with animals fed the folic acid-free diet. In fact the data of table 2 show a markedly lower growth rate for biotin- and folic acid-deficient rats than that not only for controls ($P < 0.001$), but also for folic acid-deficient rats ($P < 0.001$).

The data of table 3 indicate that the total liver content of folic acid-active substances was significantly lower in biotin-deficient rats than that in control rats ($P < 0.05$). In folic acid-deficient rats ($P < 0.0001$) and chiefly in biotin- and folic acid-deficient rats ($P < 0.001$) these decreases were much more evident.

The distribution of various folate derivatives differed in the livers of the 4 groups of animals (table 4). Significant reductions in the amounts of some derivatives, such as N^{10}- and N^5-formyltetrahydrofolic acid, N^5- and N^{10}-formyltetrahydropteroyltriglutamic acid and the derivatives of pteroylpolyglutamic acid, were observed in the livers of rats fed the biotin-deficient diet compared with those of the control rats. In folic acid-deficient rats the levels of all of the folate derivatives were decreased. These same compounds were decreased to a greater extent in the doubly deficient animals.

From the data of table 5, with respect to the conversion in vivo of folic acid to its

TABLE 5

Effects of biotin and folic acid on the conversion in vivo of folic acid to its activated forms: liver folate activities in folic acid-injected rats

Group	Treatment	Folic acid activity assays		
		P. cerevisiae	*L. casei*	*S. faecalis*
		mμg/g fresh tissue	*mμg/g fresh tissue*	*mμg/g fresh tissue*
1	Control	1,911 ± 171.3 [1]	4,631 ± 516.3	2,706 ± 217.7
2	Biotin-deficient	998 ± 101.9	2,928 ± 326.4	2,074 ± 197.3
3	Folic acid-deficient	631 ± 68.7	1,168 ± 111.4	660 ± 49.3
4	Biotin- and folic acid-deficient	369 ± 29.3	776 ± 80.7	477 ± 36.6

[1] Each value is given as the mean ± SE of mean of 8 determinations on different animals.

activated forms, it appears that in biotin-deficient rats, the liver levels of folate activities, assayed by *P. cerevisiae*, *L. casei* and *S. faecalis* after injection of folic acid were significantly lower than those of control rats ($P < 0.001$; $P < 0.02$; $P < 0.05$, respectively.) Nevertheless even lower levels were observed in the folic acid-deficient rats ($P < 0.001$) and in the biotin- and folic acid-deficient rats ($P < 0.001$).

The quantities of individual folate derivatives (table 6) differed in the livers of biotin-deficient animals. These differences were more marked in the folic acid-deficient rats and even more so in the doubly deficient rats.

In table 7 are reported the data concerning the conversion in vivo of folic acid to its activated forms measured by assaying the folate metabolites excreted by rats in the urine after injection of folic acid. In folic acid-deficient rats the "CF-regular" activity, which is the measure of N^5-formyltetrahydrofolic acid, and the "CF-aseptic" activity, which is the measure of all reduced and formylated derivatives of folic acid plus tetrahydrofolic acid, were significantly lower than for the control rats ($P < 0.005$). In the urine of biotin-deficient rats, in addition to the "CF-regular" and the "CF-aseptic" activities, the "CF" autoclaved" activity, which is the measure of N^5- and N^{10}-formyltetrahydrofolic acid and N^5-, N^{10}-methenyltetrahydrofolic acid, was markedly lower ($P < 0.01$). Further reduction of all 3 CF activities was observed in the urine of doubly deficient rats ($P < 0.001$, $P < 0.02$, and $P < 0.001$, respectively).

The quantity of citrovorum factor formed in the systems with the liver homogenate of biotin-deficient rats was significantly lower than that with liver homogenate of control rats ($P < 0.05$) (table 8). The quantity formed in systems from folic acid-deficient rats was markedly lower ($P < 0.001$). Finally, a further decrease in the quantity of citrovorum factor formed in systems from doubly deficient animals was observed ($P < 0.001$).

The data concerning the urinary excretion of FIGlu (table 9) indicate that the quantity of this metabolite of the histidine excreted by biotin-deficient rats does not appear significantly changed when

TABLE 6

Effects of biotin and folic acid on the conversion in vivo of folic acid to its activated forms: distribution of various folate derivatives in the liver of rats after injection of folic acid

Compounds	Liver concentration folate derivatives			
	Control	Biotin-deficient	Folic acid-deficient	Biotin- and folic acid-deficient
	mμg/g tissue	*mμg/g tissue*	*mμg/g tissue*	*mμg/g tissue*
N^{10}-Formyltetrahydrofolic acid	1,537 ± 141.31 [1]	722 ± 65.30	503 ± 61.34	294 ± 31.27
N^{10}-Formyltetrahydrofolic acid and N^{10}-formylfolic acid	1,097 ± 96.20	1,447 ± 131.32	239 ± 17.93	118 ± 10.80
N^5 Formyltetrahydrofolic acid	268 ± 22.70	176 ± 14.50	57 ± 4.75	33 ± 4.39
N^{10}-Formyltetrahydropteroyltriglutamic acid	1,047 ± 94.50	338 ± 31.35	253 ± 29.20	209 ± 22.50
Tetrahydrofolic acid	176 ± 12.32	122 ± 10.41	101 ± 8.12	70 ± 6.15
N^{10}-Formyl derivatives of a pteroylpolyglutamic acid	163 ± 9.75	181 ± 21.24	37 ± 2.27	63 ± 6.95
N^5-Formyltetrahydropteroyltriglutamic acid	180 ± 17.60	140 ± 11.91	26 ± 1.96	15 ± 1.61
N^5-Formyl derivatives of a pteroylpolyglutamic acid	37 ± 42.55	15 ± 1.62	11 ± 2.10	4 ± 0.54

[1] Each value is given as the mean ± SE of mean of 8 determinations on different animals.

TABLE 7

Effects of biotin and folic acid on the conversion in vivo of folic acid to its activated forms: urinary excretion of folate metabolites by rats after injection of folic acid [1]

Group	Treatment	"CF-regular" activity [2]	"CF-autoclaved" activity [3]	"CF-aseptic" activity [4]
		mμg urine/12-hr period/rat		
1	Control	1,240 ± 119 [5]	7,300 ± 824	11,820 ± 667
2	Biotin-deficient	700 ± 72	4,240 ± 222	5,100 ± 516
3	Folic acid-deficient	990 ± 102	7,210 ± 844	9,160 ± 1,035
4	Biotin- and folic-acid deficient	670 ± 28	4,960 ± 182	5,710 ± 443

[1] See Experimental section for explanation of CF-regular, -autoclaved, and -aseptic activity.
[2] "CF-regular" included: N^5-formyltetrahydrofolic acid (citrovorum factor).
[3] "CF-autoclaved" included: N^5-formyltetrahydrofolic acid, N^{10}-formyltetrahydrofolic acid and N^5,N^{10}-methenyltetrahydrofolic acid.
[4] "CF-aseptic" included: N^5-formyltetrahydrofolic acid, N^{10}-formyltetrahydrofolic acid, N^5,N^{10}-methenyltetrahydrofolic acid.
[5] Each value is given as the mean ± SE of mean of 8 determinations on different animals.

TABLE 8

Effects of biotin and folic acid on the conversion in vitro of folic acid into citrovorum factor by rat liver homogenate

Group	Treatment	Citrovorum factor formed
		mμg/g fresh tissue
1	Control	4,430 ± 465 [1]
2	Biotin-deficient	3,240 ± 214
3	Folic acid-deficient	940 ± 120
4	Biotin- and folic acid-deficient	400 ± 95

[1] Each value is given as the mean ± SE of mean of 6 determinations on different animals.

TABLE 9

Effects of biotin and folic acid in the urinary excretion of formiminoglutamic acid by rat

Group	Treatment	Formiminoglutamic acid excreted
		μmoles/7 hours/rat
1	Control	4.21 ± 0.34 [1]
2	Biotin-deficient	3.20 ± 0.30
3	Folic acid-deficient	23.80 ± 0.94
4	Biotin- and folic acid-deficient	36.87 ± 2.22

[1] Each value is given as the mean ± SE of mean of 8 determinations on different animals.

compared with that of the control rats. On the contrary the amounts excreted by folic acid-deficient rats ($P < 0.001$) and even more by biotin- and folic acid-deficient rats appear markedly higher ($P < 0.001$).

DISCUSSION

The data obtained in the present research show principally a further confirmation of the relationships between biotin and folic acid. With respect to the nature of these relationships the results appear to demonstrate that biotin is in some way involved in folic acid metabolism and in particular in its ultilization for the biosynthesis of coenzymatic derivatives. All the data support this hypothesis. The total liver content of folic acid activities and most of the various compounds, which constitute these activities, appear significantly decreased in biotin-deficient rats as compared with the control animals. In addition to the deficiency of biotin superimposed on the folic acid-deficiency effects, further significant reductions resulted in the low liver levels of folate derivatives described above. Indirect proof of the effect of biotin on the concentration of folate derivatives is shown by the fact that in doubly deficient rats the quantity of FIGlu excreted in the urine is markedly higher when compared not only with control rats but also with folic acid-deficient rats. It is known that FIGlu is a product derived from histidine

(10) and its metabolism, or more exactly, the first step of its metabolism is dependent upon the presence in tissues of folate coenzymes and in particular of tetrahydrofolic acid (11, 12). Consequently the quantity of FIGlu excreted will be higher as the tissue storage of folate coenzymes becomes lower. Therefore, the large increase in the excretion of FIGlu observed in doubly deficient rats means that in these animals the levels of folate coenzymes are lower than the level of folic acid-deficient rats.

Nevertheless a more direct demonstration of the alteration determined by biotin deficiency in the utilization of folic acid appears from the results on the conversion in vivo and in vitro of folic acid to its activated forms. In the experiments in vivo it has been observed that in biotin-deficient rats the quantity of folate metabolites excreted in the urine after administration of folic acid is significantly lower than that excreted by control rats. Also, after injection of folic acid in a quantity proportional to body weight, the levels of various folate derivatives of liver of biotin-deficient rats remain lower than those of control rats. Furthermore, in this case the double deficiency resulted in greater changes of both urinary excretion of folate metabolites, and of liver storage of various activated forms of folic acid than with the folic acid deficiency alone.

However, the data obtained in experiments in vitro correspond with those of the experiments on the intact animals. In fact the liver homogenates of biotin-deficient rats convert folic acid to citrovorum factor less effectively than those of control rats. In addition the homogenates of doubly deficient rats, show in turn, a decreased efficiency of conversion of folic acid when compared with those of folic acid-deficient rats.

Although the participation of biotin in the process of conversion of folic acid to activated reduced and formylated forms has been determined, it still is not known whether biotin has a direct coenzymatic effect or an indirect effect. However, it appears unlikely that biotin acts by increasing the tissue storage of co-factors such as ATP, NADPH, pyridoxal 5-phosphate involved, in addition to Mg^{++}, in this process. Marchetti et al. (6), in fact, observed in previous experiments that the addition of suitable amounts of these substances to reaction mixtures did not change the quantities of citrovorum factor formed in systems with liver homogenates of biotin-deficient animals.

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Initiation of Glucose 6-Phosphatase Adaptation in the Rat [1]

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ABSTRACT Rats trained to eat for only one hour each day were used to investigate the time required for increases in hepatic glucose 6-phosphatase activity to occur in response to ingestion of high sucrose and high protein diets. Glucose 6-phosphatase activity per unit of body weight decreased during the first few hours after the rats had eaten, regardless of the diet consumed. After 4 hours glucose 6-phosphatase activity of rats fed high protein or high sucrose diets was greater than that of rats fed a 25% casein diet containing dextrin. There was a continuous increase in liver glycogen concentration and relative size in all groups up to 8 hours after they had eaten. Hydrocortisone injection produced a marked increase in liver glycogen concentration and relative size of rats within 8 hours after injection, but had no effect on the glucose 6-phosphatase activity per unit of body weight 8 or 12 hours after injection.

Certain dietary treatments (high protein, high sucrose) cause a significant increase in liver glucose 6-phosphatase activity [2] per unit of body weight in the rat. The increase, which occurs within 24 hours, appears to be a response to a drastic reduction in the amount of glucose in the diet or to the substitution of fructose for a portion of the glucose and has been interpreted as a metabolic adaptation (1). Two types of glucose 6-phosphatase adaptation to dietary alterations have been observed (2). First, a permanent type in which the greater activity persists as long as the stimulus is continued; this occurs when rats are fed a diet high in fructose or sucrose. Second, a temporary rapid increase in glucose 6-phosphatase activity with a return to normal after several days even if the stimulus is continued; this occurs when rats are fed a high protein diet. Injections of adrenal glucocorticoid hormones have also been shown to increase glucose 6-phosphatase activity in the rat (3). Since glucose 6-phosphatase activity usually reaches a maximum within 24 hours after institution of the dietary treatments, an effort has been made to determine the minimal length of time required for high protein and high sucrose diets and hydrocortisone injections to cause a response in glucose 6-phosphatase relative to the time required for them to cause changes in liver glycogen content and relative liver size.

EXPERIMENTAL

Animals and diets. Male rats of the Sprague-Dawley strain, weighing between 200 and 300 g, housed in individual, suspended, screen-bottom cages were used in all experiments. Animals used for the hormonal studies were fed ad libitum; all others were fed for only a single interval daily. The latter were treated in the following manner. They were offered unlimited food for one hour only, at the same time each day. After an initial loss in weight, the animals showed a consistent gain in weight and after 2 weeks could ingest nearly as much food in the one-hour period as rats of the same weight fed ad libitum could ingest in 24 hours. On the day of the experiment the various groups were fed in a time sequence that made it possible to remove each group of animals at exactly the same time after the diets were offered. The animals used for the hormonal studies were fed a "dextrin diet" ad libitum for at least one week prior to the experiment. On the day of the experiment the various groups were injected in

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[2] Liver glucose 6-phosphatase activity, indicates activity per gram of liver; glucose 6-phosphatase activity per unit of body weight, total liver glucose 6-phosphatase activity times 100 divided by the body weight; specific activity, liver glucose 6-phosphatase activity per gram of liver protein; relative liver weight, weight of the liver times 100 divided by the body weight.

sequence at intervals sufficiently long to permit each group of animals to be used at exactly the same time after injection. Control animals were injected intraperitoneally with isotonic saline, 1 ml/100 g of body weight; and the hydrocortisone-treated animals with hydrocortisone acetate in isotonic saline (5 mg of hydrocortisone acetate/1 ml), 1 ml/100 g of body weight.

Three diets were used throughout this study. These were: the "dextrin diet" containing 65% dextrin, 25% casein and 5% corn oil; the "sucrose diet" containing 65% sucrose, 25% casein and 5% corn oil; the "high protein diet" containing 90% casein and 5% corn oil. All diets contained sufficient salts and vitamins (4) in addition to the aforementioned ingredients. All animals were fed the dextrin diet until the time of the experiment, which included a minimum of a 2-week training period for those fed for only one hour daily.

Assay procedure. The rats were killed at various lengths of time after being offered the diets or after the injections. The livers were carefully and completely removed, weighed, and placed in ice. A portion of the chilled liver was weighed on a torsion balance to the nearest milligram, and homogenized at zero to 4° in 40 volumes of 0.1 M potassium citrate buffer, pH 6.5. The homogenate was filtered through cheesecloth and the filtrate used for the glucose 6-phosphatase assay and protein determination. Another accurately weighed portion of the liver was used for the determination of glycogen (5). A portion of the homogenate was diluted for the protein determination (6). For the glucose 6-phosphatase assay 0.1 ml of 0.06 M dipotassium glucose 6-phosphate, pH 6.5, was added to 0.1 ml of the homogenate both of which had been preincubated at 37.5° for 5 minutes. After incubation at 37.5° for 15 minutes the reaction was stopped by adding 2 ml of 10% trichloroacetic acid. The activity was estimated by measuring the amount of inorganic phosphate released (7). These assay conditions produced zero order kinetics for values up to twice the greatest values reported.

Enzyme unit. The amount of enzyme which causes the release of 1 μmole of inorganic phosphate from glucose 6-phosphatase in one minute at 37.5° and pH 6.5 is defined as one unit of glucose 6-phosphatase.

RESULTS

Values for glucose 6-phosphatase activity, liver glycogen concentration, and relative liver size of rats trained to eat for only one hour daily and killed at various time-intervals after they had been fed either the dextrin or the sucrose diet are shown in table 1. Since all rats received the dextrin diet prior to the time of the experiment, the zero-time value also serves as a 24-hour value for the dextrin group. One and two

TABLE 1

Initiation of glucose 6-phosphatase adaptation in rats trained to eat for only 1 hour daily and fed a diet high in sucrose

Diet	Hours after eating	Glucose 6-phosphatase activity			Glycogen	Relative liver size	Avg food consumption
		units/100 g body wt	*units/g liver*	*units/g liver protein*	%	*g*	*g*
	0	69 ± 4.8 [1]	21.7	108	1.7	3.28	—
Dextrin	1	60 ± 3.0	19.5	107	1.9	3.12	10.3
Sucrose	1	60 ± 3.8	19.9	110	2.4	3.04	6.7
Dextrin	2	55 ± 1.9	16.5	96	2.8	3.38	9.8
Sucrose	2	55 ± 3.8	17.0	98	3.6	3.25	8.1
Dextrin	4	47 ± 3.3	13.6	79	4.0	3.56	11.2
Sucrose	4	56 ± 2.2 [2]	15.7	93	5.2	3.52	7.0
Dextrin	8	50 ± 2.5	13.7	78	4.5	3.60	10.4
Sucrose	8	67 ± 2.2 [3]	16.8	92	6.4	3.95	8.3
Dextrin	24	69 ± 4.8	21.7	108	1.7	3.28	—
Sucrose	24	100 ± 3.8 [3]	28.2	127	0.3	3.10	—

[1] SE of mean for 7 animals.
[2] $P < 0.05$, sucrose group vs. dextrin group for the same interval.
[3] $P < 0.01$, sucrose group vs. dextrin group for the same interval.

TABLE 2

Initiation of glucose 6-phosphatase adaptation in rats trained to eat for only 1 hour daily and fed a diet high in protein

Diet	Hours after eating	Glucose 6-phosphatase activity			Glycogen	Relative liver size	Avg food consumption
		units/100 g body wt	*units/g liver*	*units/g liver protein*	%	g	g
Dextrin	2	62 ± 2.2 [1]	18.9	101	3.0	3.27	10.5
Protein	2	59 ± 2.1	20.5	94	< 0.1	2.95	5.7
Dextrin	4	58 ± 3.0	18.3	93	4.1	3.15	12.7
Protein	4	66 ± 3.0 [2]	21.0	106	0.2	3.16	6.9
Dextrin	8	57 ± 3.0	16.1	79	3.2	3.43	7.2
Protein	8	69 ± 1.2 [3]	19.2	99	0.7	3.56	4.0

[1] SE of mean for 5 animals.
[2] $P < 0.05$, high-protein group vs. dextrin group for the same interval.
[3] $P < 0.01$, high-protein group vs. dextrin group for the same interval.

hours after eating there was essentially no difference between the glucose 6-phosphatase activities of the 2 groups, expressed as activity per unit of body weight, per gram of liver, or per gram of liver protein. The values for animals fed both diets showed similar decreases at 2 hours but the glucose 6-phosphatase activity of animals fed the dextrin diet decreased considerably below that for animals fed the sucrose diet by 4 hours. In contrast with previous experiments on rats fed ad libitum, in which the glucose 6-phosphatase adaptation in the group fed the sucrose diet was observed as an increase above the value for the group fed the dextrin diet (1), with rats fed for only one hour daily, the value for the group fed the dextrin diet decreased, whereas that for the group fed the sucrose diet remained constant after 2 hours. A significant difference between the values for the 2 groups was taken as evidence of a glucose 6-phosphatase adaptation. Four hours after the animals had eaten, values for glucose 6-phosphatase activity per unit of body weight, per gram of liver and specific activity for rats fed the sucrose diet were significantly higher than those for animals fed the dextrin diet; the difference per unit of body weight was greater at 8 hours and still greater at 24 hours. Glucose 6-phosphatase activity of the dextrin group leveled off between 4 and 8 hours, then increased by 24 hours. The specific activity values for the dextrin groups at 4 and 8 hours were comparable to values reported for animals fed the dextrin diet ad libitum.

Both groups showed a steady increase in relative liver size [3] with increasing time after the feeding period, at least for the first 8 hours; animals fed the sucrose diet showed a more rapid increase than those fed the dextrin diet; similar observations have been made on rats fed the sucrose diet ad libitum (8). Also during the first 8 hours the liver glycogen content of both groups showed a steady increase, with animals fed the sucrose diet showing a more rapid increase than those fed the dextrin diet. A greater accumulation of liver glycogen was also observed when rats were fed a fructose diet ad libitum (8). The total liver glycogen of the rats fed for only one hour daily increased more than the liver glycogen concentration indicates because there was also an increase in relative liver size.

It is improbable that the differences in food consumption influenced the results up to 8 hours as all animals, taken up to this time including those fed the high protein diet (table 2), had an appreciable amount of food remaining in their stomachs when they were killed.

Values for glucose 6-phosphatase activity, liver glycogen concentration, and relative liver size of rats killed at various time-intervals after they had been fed the dextrin diet or the high protein diet are shown in table 2. At the 4-hour interval the glucose 6-phosphatase activities per unit of body weight and per gram of liver for the dextrin and the high protein groups

[3] See footnote 2.

were different. The glucose 6-phosphatase specific activity of the high protein group, which was lower than that of the dextrin group at 2 hours, was greater than that of the dextrin group at 4 hours. As in experiments in which sucrose was used to induce an adaptation, at 8 hours there was a significant difference between the glucose 6-phosphatase values for the dextrin and the high protein group. The glucose 6-phosphatase specific activity of the high protein group remained high but that of the dextrin group fell to within the range usually found for rats fed the dextrin diet ad libitum. The liver glycogen content of the dextrin group remained significantly higher than that of the high protein group; however, the value for the high protein group increased from the second to the eighth hour.

The effects of hydrocortisone injections on glucose 6-phosphatase activity, liver glycogen, and relative liver size are shown in table 3. Values for glucose 6-phosphatase activity for the groups injected with saline or with hydrocortisone were not significantly different at 8 or 12 hours although triamcinolone, which appears to be a more potent glucocorticoid than hydrocortisone, has been reported to increase liver glucose 6-phosphatase activity 6 hours after administration (9). Since liver glycogen concentration and relative liver size increased by 8 hours in the hydrocortisone-treated groups, it seems evident that the hydrocortisone was absorbed. Absorption should have been continuous over the 12-hour period as solid flecks of hydrocortisone were observed in the peritoneal cavity of all the hydrocortisone-treated animals when they were killed.

To determine whether the observed decrease in glucose 6-phosphatase activity after the feeding period was related to weight change, nutrient absorption, or food intake, rats that were fed the dextrin diet or gum arabic during the usual feeding period, or were kept without food, were killed 2 hours later for determination of glucose 6-phosphatase, glycogen and protein. Results based upon pre-feeding, initial weight and final weight are shown in table 4. A decrease in liver glucose 6-phosphatase activity occurred only in the rats that consumed a utilizable foodstuff. This was apparent whether the calculations were based on initial or final body weight.

DISCUSSION

Rats trained to eat for only one hour daily are convenient for studying over short time-intervals the effects of dietary alterations on liver glucose 6-phosphatase activity. Glucose 6-phosphatase responses to both the sucrose and the high protein diets appear to start between 2 and 4 hours after the ingestion of the diet since there were measurable differences between the glucose 6-phosphatase activity per unit of body weight of the experimental groups and the control (dextrin) group within 4 hours. These differences have been interpreted as enzymatic adaptations and appear to be due to increase in or maintenance of glucose 6-phosphatase activity in the high protein and sucrose groups, whereas the activity of the control group decreases. The decrease in the activity of

TABLE 3

Effect of hydrocortisone injections on liver glucose 6-phosphatase activity, glycogen, and relative liver size of rats fed dextrin basal diet ad libitum

Injection	Hours after injection	Glucose 6-phosphatase activity			Glycogen	Relative liver size
		units/100 g body wt	*units/g liver*	*units/g liver protein*	%	*g*
Saline	8	71 ± 3.6 [1]	15.9	87	3.5 ± 0.4	4.5 ± 0.1
Hydrocortisone	8	70 ± 3.8	13.2	81	6.8 ± 0.7 [2]	5.3 ± 0.1 [2]
Saline	12	72 ± 3.7	14.8	95	4.6 ± 0.8	4.9 ± 0.1
Hydrocortisone	12	77 ± 1.8	12.9	88	6.8 ± 0.4 [3]	6.0 ± 0.2 [2]

[1] SE of mean for 5 animals.
[2] $P < 0.01$, group injected with hydrocortisone vs. group injected with isotonic saline for same time interval.
[3] $P < 0.05$, group injected with hydrocortisone vs. group injected with isotonic saline for same time interval.

TABLE 4

Effect of food intake on liver glucose 6-phosphatase activity of rats trained to eat for only 1 hour daily [1]

Group	1	2	3
Diet	No food	Gum arabic	Dextrin
Original wt	240	240	240
Final wt	237	245	265
Food consumption	—	4.3	9.5
Relative liver size	2.9	2.9	3.4
Glucose 6-phosphatase, units/g liver	24.3	23.5	15.4
Glucose 6-phosphatase, units/100 g original body wt	70 ± 4 [2]	69 ± 4	57 ± 4
Glucose 6-phosphatase, units/100 g final body wt	71 ± 4	68 ± 4	53 ± 4
Glucose 6-phosphatase, units/g liver protein	111	109	83
Liver protein, mg/g liver	218	220	186
Glycogen, %	0.11	0.11	4.92
Glycogen, mg/100 g body wt	32	40	1682
Protein, mg/100 g body wt	636	636	636
Protein, mg/100 g original body wt	628	650	686

[1] All values are the average for 4 rats killed 2 hours after being fed.
[2] SE of mean.

the control group was not due to an insufficient intake of protein because the total liver protein per unit of body weight increased at about the same rate in animals fed the dextrin diet as in those fed the sucrose and high protein diets.

The decrease in specific activity of glucose 6-phosphatase and the increase in total liver protein of the dextrin group during the first 4 hours after eating indicates that overall liver protein synthesis was proceeding at a more rapid rate than the synthesis of glucose 6-phosphatase. The specific activity remained constant from the fourth to the eighth hour after eating indicating that during this period glucose 6-phosphatase was being synthesized at the same rate as overall liver protein. The specific activity of the sucrose and the high protein groups remained fairly constant from the second to the eighth hours after eating, thus maintaining the ratio of glucose 6-phosphatase to liver protein observed at the end of 2 hours. This would occur if the rate of synthesis of glucose 6-phosphatase were the same as that of overall liver protein after 2 hours.

These results are complicated by changes that are apparently due to the feeding technique. The decrease in glucose 6-phosphatase activity in all 3 groups during the first 2 hours after ingestion of the diets appears to be a response to the ingestion of utilizable foodstuff regardless of its composition. The decrease in glucose 6-phosphatase activity along with the increases in glucokinase after carbohydrate feeding (10) might facilitate a greater and more rapid deposition of liver glycogen. Substantial amounts of glycogen were deposited in the livers of animals other than those fed the high protein diet during a short period of time. In the high protein group a lack of substrate, namely hexose phosphate, was probably responsible for this effect since the length of time after the ingestion of the diet was fairly short. The increase in glucose 6-phosphatase activity of the dextrin group 24 hours after ingestion of the diet could be part of a mechanism which facilitates the utilization of liver glycogen stores at this time.

The effect of the composition of the diet being fed is superimposed on the changes due to the feeding procedure. The adaptation first becomes evident 4 hours after the ingestion of the sucrose diet or the high protein diet, and becomes more evident after 8 hours. This adaptation is comparable to the glucose 6-phosphatase adaptation in rats fed ad libitum (1). More rapid formation of liver glycogen by the sucrose group than the dextrin group was also observed in rats fed ad libitum (8).

It appears unlikely that the glucose 6-phosphatase adaptation is caused by adrenal glucocorticoids as there was no difference in glucose 6-phosphatase activity per unit of body weight between the hydrocortisone-injected and control groups at

either 8 or 12 hours. Also increases in glucose 6-phosphatase activity have been shown to occur in adrenalectomized rats as a result of dietary manipulations (11). The slight difference in specific activity between the 2 groups may be due to a higher ratio of structural to enzyme protein in the larger livers of the hydrocortisone-treated group.

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Dietary Fat and Fatty Acid Compositions of Rat Leucocytes and Granules [1]

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ABSTRACT To study the effect of dietary fats on fatty acid constituents of polymorphonuclear leucocyte and granule lipids, rats were fed diets which contained either 15% corn oil, 15% hydrogenated coconut oil, or no fat for 40 weeks, and polymorphonuclear leucocytes were separated from the peritoneal exudate after injection of a saline containing glycogen. The dietary alterations did not significantly influence the percentages of lipid components in the leucocytes and granules, but their fatty acid patterns showed a marked response. A lower content of linoleic and arachidonic acids in leucocyte and granule lipids from rats fed essential fatty acid-deficient diets, a hydrogenated coconut oil or a fat-deficient diet, was accompanied by a higher content of palmitoleic, oleic, and eicosatrienoic acids. The fatty acid patterns of both granule phospholipids and non-phospholipids responded to the dietary alterations to a lesser degree than total leucocyte lipids.

On the basis of cytochemical observations, association of lipids with polymorphonuclear (PMN) leucocytes and their subcellular fractions has long been accepted. Lillie and Burtner (1) showed these lipids to be largely protein-bound. Over 60% of the total leucocyte lipids was present as phospholipids (2). Thus, leucocytes and their subcellular particles were considered to have surrounding lipoprotein membranes, with phospholipids as the main lipid component. PMN leucocytes have also been shown to be very active lipid synthesizers, rapidly incorporating acetate, labeled glucose, and phosphate into lipid fractions (3). Furthermore, Elsbach (4) has demonstrated rapid incorporation of free fatty acids into cell lipids. It is possible that a change in dietary fat may alter the type of fatty acids incorporated into leucocyte lipids, which in turn affects membrane properties of leucocytes and their granules. The latter are known to be rich in a variety of hydrolytic enzymes and to possess properties and functions very similar to those of hepatic lysosomes (5). In the present study an attempt was made to detect the effect of dietary fat on fatty acid constituents of PMN leucocyte and granule lipids.

MATERIALS AND METHODS

Ninety male weanling rats were divided into 3 groups of 30 each and were fed diets (table 1) which contained 15% corn oil, 15% hydrogenated coconut oil (table 2), or no fat for 40 weeks, at which time the average weights of the rats fed these diets were 615, 512, and 460 g, respectively.

TABLE 1
Composition of basal diet

	g/kg
Casein, vitamin-free	200
Glucose [1]	607
Salts mix W [2]	40
Fat (or glucose)	150
Choline chloride	2
	mg/kg
Niacin	100
Inositol	100
Folic acid	2
Ca pantothenate	60
Biotin	0.5
p-Aminobenzoic acid	100
Thiamine·HCl	20
Riboflavin	20
Pyridoxine·HCl	10
Vitamin B_{12}	0.005
Menadione	10
dl-α-Tocopherol	100
	IU/kg
Vitamin A	24,000
Vitamin D	2,400

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Wesson, L. G. Science, 75: 339, 1932.

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TABLE 2

Fatty acid composition of the dietary fats [1]

Identity	Corn oil	Hydrogenated coconut oil
8:0	—	1.0
10:0	—	5.0
12:0	tr [2]	50.7
14:0	0.5	19.0
14:1	0.1	—
16:0	11.5	9.9
16:1	0.1	0.1
18:0	2.8	10.4
18:1	30.1	2.8
18:2	54.1	0.6
18:3	0.5	—
20:0	tr	0.3
20:1	0.4	0.4

[1] Expressed as a percentage of the total fatty acids recovered by gas-liquid chromatography.
[2] Trace, less than 0.1% detected.

The rats were then injected intraperitoneally with 50 ml of 0.9% sterile saline containing 50 mg of glycogen. Eighteen hours after the injection, the animals were killed and the peritoneal cavities washed 3 times with ice-cold 0.9% saline. Stained smears and wet-mount differential counts of leucocytes in the exudate showed the population of PMN leucocytes to be 98% or more. Separation of PMN leucocytes from the peritoneal exudate and the isolation of the leucocyte granules were carried out essentially by the method of Cohn and Hirsch (5).

For the extraction of lipids, leucocyte homogenate and isolated granule suspension in 0.34 M sucrose were lyophilized, crushed to a fine powder, and extracted with 2:1 chloroform-methanol according to the method of Folch et al. (6). Fractionation of total lipids into neutral lipids and phospholipids was conducted by chromatographic procedures of Lis et al. (7). Gravimetric determination of total lipids, non-phospholipids and phospholipids was carried out by removing the solvent at reduced pressure and drying to constant weight over anhydrous phosphopentoxide at 0°.

To determine the fatty acid composition of leucocyte and granule lipids, methyl esters were prepared from the lipids by transesterification with 5% methanolic HCl (8) and were analyzed by gas-liquid chromatography on a Model 600-B Aerograph equipped with a hydrogen flame detector. The 2 types of stationary phase used were the 60–80 mesh 15% ethylene glycol succinate (EGS) and the 20% Apiezon M. The oven temperatures were 175° for the EGS and 225° for the Apiezon M. The carrier gas was nitrogen, which was applied to the EGS and Apiezon M columns at a pressure of 10 psi and 16 psi, respectively.

The fatty acid peaks were identified by making comparisons with known standards and by calculation of carbon numbers based on relative retention times plotted on semilogarithmic paper. The use of both non-polar (EGS) and polar (Apiezon M) stationary phases facilitated reliable identification of most fatty acids (9). Occasional complete hydrogenation was carried out to provide additional information for identification of peaks (10). Separation of dimethyl acetals of long-chain aldehydes from the fatty acid methyl esters was conducted by the saponification method described by Farquhar (11).

For quantitation, the areas of individual peaks were obtained by the triangulation method and expressed as a percentage of the total area.

TABLE 3

Lipid content of granules and whole leucocytes

Fraction	Dietary fat	Total lipid [1]	Non-phospholipids [2]	Phospholipids [2]
Granules	15% Corn oil	8.1 ± 3.7	42.7 ± 8.7	57.3 ± 9.3
	15% Hydrogenated coconut oil	7.5 ± 4.0	44.2 ± 7.2	55.8 ± 8.9
	Fat-deficient	7.9 ± 3.5	44.0 ± 9.1	56.0 ± 7.8
Whole leucocytes	15% Corn oil	12.6 ± 2.9	34.8 ± 3.7	64.8 ± 4.3
	15% Hydrogenated coconut oil	13.9 ± 2.1	36.0 ± 2.5	63.8 ± 5.7
	Fat-deficient	12.1 ± 3.5	34.2 ± 9.1	65.8 ± 6.8

[1] Expressed as a percentage of the dry weight of granules; ± SD of mean.
[2] Expressed as a percentage of the weight of the total lipid.

RESULTS

The feeding for a period of 40 weeks of diets which contained either 15% corn oil, 15% hydrogenated coconut oil, or no fat to rats did not significantly influence the total lipid, phospholipid, and non-phospholipid content of leucocytes and granules (table 3). However, the fatty acid composition of leucocyte lipids was influenced by the dietary alterations (table 4). The leucocyte lipids from rats fed corn oil contained approximately 30 times more linoleic acid and 3 times more arachidonic acid than those from 2 groups of rats that had received essential fatty acid-deficient diets. The lower content of linoleic and arachidonic acids in the lipids from these groups of rats was accompanied by higher concentrations of palmitoleic, oleic, and eicosatrienoic acids. Oleic acid constituted 30% of the total fatty acid content in the rats deficient in the essential fatty acids (EFA). Palmitic acid showed a relatively constant level regardless of the dietary variations. The stearic acid content, however, was significantly lower in the EFA-deficient rats.

The fatty acid patterns of both phospholipids (table 5) and non-phospholipids (table 6) from granule lipids responded to dietary factors in a manner similar to that observed for the total lipids of leucocytes, although the degree of response was less pronounced in the granule lipids. An especially noticeable difference was observed in the linoleic acid content. The diet which contained corn oil caused significantly less linoleic acid to accumulate in the phospholipids and neutral lipids of granules than in total leucocyte lipids, whereas EFA-deficient diets caused a less extensive reduction of linoleic acid in both granule lipids. The distribution of fatty acids in granule phospholipids and granule non-phospholipids was very similar. However, the arachidonic acid content in granule phospholipids from EFA-deficient rats was significantly higher than in granule non-phospholipids.

DISCUSSION

In the present study, a considerable change in the fatty acid composition of lipids from granule particles and unfrac-

TABLE 4

Fatty acid composition of total lipids from whole leucocytes [1]

Identity [2]	Dietary fat		
	15% Corn oil	15% Hydrogenated coconut oil	Fat-deficient
12:0	1.3 ± 0.2	2.6 ± 0.2	2.2 ± 0.9
14:0 ald	tr [3]	0.1 ± 0.1	0.2 ± 0.2
14:0	2.1 ± 0.1	2.0 ± 0.6	2.0 ± 0.5
15:0	1.8 ± 0.4	0.2 ± 0.1	0.1 ± 0.1
16:0 ald	tr	2.0 ± 1.5	0.9 ± 0.7
16:0	23.4 ± 0.6	23.5 ± 0.8	23.8 ± 1.1
16:1	1.0 ± 0.2	5.3 ± 0.1	5.8 ± 0.8
17:0	tr	tr	tr
18:0 ald	1.8 ± 0.8	1.4 ± 1.0	0.9 ± 0.6
18:0	19.3 ± 0.7	8.7 ± 1.0	9.1 ± 1.5
18:1	18.0 ± 0.5	30.1 ± 2.5	32.8 ± 2.7
18:2	16.6 ± 0.8	0.5 ± 0.3	0.5 ± 0.4
20:0	0.1 ± 0.1	tr	tr
20:1	0.4 ± 0.1	0.7 ± 0.3	0.9 ± 0.3
20:2	1.0 ± 0.2	tr	tr
20:3	1.9 ± 0.1	12.3 ± 1.8	12.2 ± 1.0
20:4	10.2 ± 1.5	3.1 ± 0.5	2.7 ± 0.3
22:1	tr	1.2 ± 0.9	1.0 ± 0.7
22:3	0.1 ± 0.1	1.8 ± 1.1	1.9 ± 1.0
24:0	0.9 ± 0.5	tr	tr
24:1	0.8 ± 0.4	2.5 ± 1.9	1.6 ± 0.9
22:5	1.1 ± 0.7	0.8 ± 0.5	0.5 ± 0.3
22:6	1.9 ± 1.5	0.8 ± 0.6	1.0 ± 0.8

[1] Expressed as a percentage of the total fatty acids and aldehydes recovered; ± SD of mean.
[2] Fatty aldehydes are designated by the suffix *ald.*
[3] Trace, less than 0.1% detected.

TABLE 5

Fatty acid composition of phospholipids from the granule fraction [1]

Identity [2]	Dietary fat		
	15% Corn oil	15% Hydrogenated coconut oil	Fat-deficient
12:0	1.6 ± 0.3	1.7 ± 0.5	1.0 ± 0.3
14:0 ald	0.2 ± 0.1	0.3 ± 0.3	0.2 ± 0.1
14:0	1.4 ± 0.6	1.9 ± 0.4	1.6 ± 0.5
15:0	0.2 ± 0.1	0.2 ± 0.2	0.5 ± 0.2
16:0 ald	0.9 ± 0.4	3.0 ± 1.2	1.9 ± 0.7
16:0	22.8 ± 1.9	24.3 ± 2.3	24.0 ± 1.3
16:1	1.8 ± 0.8	4.7 ± 1.6	3.5 ± 0.9
17:0	tr [3]	tr	tr
18:0 ald	0.9 ± 0.5	0.2 ± 0.1	0.5 ± 0.3
18:0	23.1 ± 0.7	18.8 ± 0.6	18.0 ± 1.8
18:1	20.0 ± 1.5	22.4 ± 0.5	24.7 ± 1.0
18:2	10.1 ± 2.0	1.9 ± 0.7	1.7 ± 0.3
20:0	0.1 ± 0.1	tr	tr
20:1	0.1 ± 0.1	0.4 ± 0.4	0.3 ± 0.2
20:2	0.8 ± 0.1	tr	tr
20:3	1.7 ± 0.6	11.4 ± 1.2	11.2 ± 1.9
20:4	10.0 ± 0.3	6.7 ± 0.9	8.3 ± 0.4
22:1	tr	0.1 ± 0.1	0.1 ± 0.1
22:3	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.6
24:0	0.4 ± 0.3	tr	tr
24:1	0.9 ± 0.6	1.3 ± 0.2	1.2 ± 0.1
22:5	1.9 ± 0.9	0.1 ± 0.1	0.2 ± 0.2
22:6	0.6 ± 0.3	0.3 ± 0.2	0.8 ± 0.2

[1] Expressed as a percentage of the total fatty acids and aldehydes recovered; ± SD of mean.
[2] Fatty aldehydes are designated by the suffix *ald*.
[3] Trace, less than 0.1% detected.

TABLE 6

Fatty acid composition of non-phospholipids from the granular fraction

Identity [2]	Dietary fat		
	15% Corn oil	15% Hydrogenated coconut oil	Fat-deficient
12:0	1.5 ± 0.4	3.0 ± 0.6	1.9 ± 0.2
14:0 ald	tr [3]	tr	tr
14:0	2.8 ± 0.7	3.5 ± 0.7	1.6 ± 0.6
15:0	0.2 ± 0.2	0.2 ± 0.1	0.4 ± 0.2
16:0 ald	1.2 ± 0.4	1.7 ± 0.7	2.3 ± 0.6
16:0	25.9 ± 0.7	26.1 ± 1.0	25.1 ± 2.3
16:1	1.0 ± 0.3	3.5 ± 2.1	4.5 ± 0.5
17:0	tr	tr	tr
18:0 ald	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 1.0
18:0	22.1 ± 1.5	14.3 ± 0.9	13.7 ± 1.5
18:1	19.8 ± 0.8	25.5 ± 1.1	26.6 ± 2.1
18:2	9.1 ± 1.6	1.5 ± 0.4	2.1 ± 0.2
20:0	tr	tr	tr
20:1	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
20:2	1.0 ± 0.1	tr	tr
20:3	1.4 ± 0.6	10.8 ± 0.9	12.7 ± 1.2
20:4	9.8 ± 0.3	3.9 ± 0.4	4.5 ± 0.6
22:1	tr	0.6 ± 0.2	0.2 ± 0.2
22:3	0.3 ± 0.2	1.6 ± 0.7	1.2 ± 0.3
24:0	0.6 ± 0.1	tr	tr
24:1	0.5 ± 0.3	2.2 ± 0.7	1.3 ± 0.1
22:5	1.3 ± 0.6	0.4 ± 0.4	0.3 ± 0.2
22:6	0.8 ± 0.4	0.5 ± 0.3	0.8 ± 0.6

[1] Expressed as a percentage of the total fatty acids and aldehydes recovered; ± SD of mean.
[2] Fatty aldehydes are designated by the suffix *ald*.
[3] Trace, less than 0.1% detected.

tionated PMN leucocytes was induced by varying the dietary fat. The fatty acid composition of PMN leucocyte and granule lipids obtained from rats fed hydrogenated coconut oil or a fat-deficient diet showed a typical pattern of EFA deficiency with a notable increase in eciosatrienoic ($C_{20:3}$) acid. Low concentrations of this acid are generally associated with adequate levels of dietary EFA (12, 13).

The increases in the concentrations of palmitoleic and oleic acids, in addition to the higher concentration of eicosatrienoic acid in the EFA-deficient animals, appear to reflect the tendency for the animals to balance the ratio of saturated to unsaturated fatty acids, and the leucocytes do not present any unusual features in this respect. Such a tendency is known to be the result of accelerated synthesis of unsaturated acids from palmitic and stearic acids.

The similar response of the leucocyte fatty acid patterns to hydrogenated coconut oil and fat-deficient diets indicated that ingested saturated fat, unlike unsaturated fat, does not exert any profound effect on the leucocyte fatty acids of the rats fed a fat-deficient diet. However, all long-chain fatty acids of leucocyte lipids of rats fed a fat-deficient diet should have been derived from the addition of one or more acetyls to pre-existing endogenous acyl residues of differing degrees of saturation and chain lengths, presumably by the elongation mechanism (14).

The present study also indicated that the fatty acid composition of granule phospholipids as well as non-phospholipids is altered extensively, but to a lesser degree than that of total leucocyte lipids. It is reasonable to assume that the fatty acid patterns of leucocytes and their subcellular fractions may be determined primarily by the type of fatty acids available in the bone marrow, where leucocytes are known to be produced (15). The synthesis of fatty acids in the pre-formed leucocytes (2, 3) and the active transport of free fatty acids from fatty acid-albumin complex in plasma to leucocytes and their subsequent incorporation into leucocyte lipids (4) may exert a secondary effect on the fatty acid composition of the leucocytes. The feeding of a high fat diet may depress the fatty acid synthesis, and hence the active transport of plasma free fatty acids may become of primary importance in the secondary change of leucocyte fatty acids.

It is possible that the composition of plasma free fatty acids may resemble more closely the fatty acid patterns of dietary fats than the fatty acid composition of leucocyte lipids. Furthermore, the uptake of various free fatty acids by PMN leucocytes appeared to be influenced primarily by their relative concentrations in the medium (4). Therefore, the greater response of the fatty acid composition of whole leucocyte lipids than that of granule lipids to dietary fats may suggest that the plasma free fatty acids are preferentially incorporated into the membrane lipids of leucocytes and their subcellular organelles, and not into the granules.

A somewhat different situation may exist in the case of the fat-deficient diet. The fatty acid composition of pre-formed leucocytes of rats fed a fat-deficient diet may be altered primarily by the fatty acids synthesized *in situ*. The higher concentration of linoleic and arachidonic acids in granule lipids than in total leucoctye lipids indicated that the synthesized fatty acids may be incorporated to a lesser extent in granule lipids, thus minimizing the dilution of essential fatty acids originally present in granules.

Although the processes involved in the formation of granules in PMN leucocytes have not yet been clarified, once granules are formed, the lipid constituents of the membrane may not participate in rapid turnover or active exchange with intra- and extracellular lipids as do the lipid constituents of other subcellular fractions and cell membrane. The exact nature of lipid metabolism in PMN leucocytes and the possible effect of fatty acid constituents of granule and cell membrane lipids on phagocytic processes await future investigations.

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Quantitative Evaluation of the Tryptophan, Methionine and Lysine Needs of Adult Swine for Maintenance

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ABSTRACT Nongravid gilts averaging 145 kg were used to determine maintenance (1 g nitrogen retention/day) and nitrogen equilibrium requirements for tryptophan, methionine and lysine. Nitrogen balance increased linearly with levels of each of these amino acids. Urinary nitrogen derived from urea and ammonia decreased, but that derived from creatinine remained constant, as the amino acid level increased from zero to adequacy. From 80 to 90% of the total urinary nitrogen could be accounted for in these 3 components. Expressed as mg/day/kg$^{3/4}$ the requirements for maintenance were: tryptophan, 5; methionine, 26 and lysine, 25. The adequacy of the determined maintenance requirements was tested by feeding diets containing the 7 essential amino acids at their calculated requirement levels, with graded levels of glutamic acid (nonessential amino nitrogen). A linear response to glutamic acid resulted, and the observed maximal nitrogen balance was only slightly short of the predicted balance of 1 g/day. Cystine could supply 94% of the maintenance requirement for total sulfur-bearing amino acids.

The dispensability of arginine, histidine and leucine and the indispensability of threonine, isoleucine, valine and phenylalanine for maintenance of the adult nongravid gilt have been reported previously (1, 2). It is important to know the requirements for other amino acids, particularly those most frequently found deficient in common foods and feeds. Furthermore, some measure of the amino nitrogen needs (including both indispensable and dispensable amino nitrogen and the relation of one to the other) for various physiological functions has been neglected and deserves attention.

This paper extends the investigation of the maintenance requirements of the adult female porcine by presenting nitrogen balance data from which the requirements for tryptophan, methionine and lysine were computed. In addition, source and level of dietary amino nitrogen were studied.

EXPERIMENTAL PROCEDURE

Selection of gilts, the basal purified diet used and analyses of feed, feces and urine for total nitrogen were as described previously (1, 2). Crossbred gilts weighing approximately 145 kg were used. Collection procedures were similar except that a 10-day assay period (with total urine collection being made on the last 5 days) was used here rather than the 14-day period used previously.

A Latin square design (3 gilts, 3 periods and 3 levels) was used to assay the tryptophan and methionine requirements. A randomized complete block design was used for lysine. The requirement for these 3 amino acids was found from the relation of nitrogen balance to amino acid intake, determining the intersect of the line with daily nitrogen balances of zero (nitrogen equilibrium requirement) and 1 g (adopted maintenance requirement). After the maintenance requirement for the indispensable amino acids had been determined, a diet devoid of arginine, histidine and leucine but with each indispensable amino acid at its calculated requirement for maintenance was fed with 3 levels of glutamic acid.

A partial fractionation of urinary nitrogen was carried out on urine samples from the tryptophan, methionine and lysine as-

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says by determining nitrogen derived from urea and ammonia as well as that derived from creatinine. Urea plus ammonia nitrogen was determined by the colorimetric method of Fawcett and Scott (3). Creatinine was determined according to procedures outlined by Hawk et al. (4).

RESULTS AND DISCUSSION

Tryptophan. Nitrogen balance was significantly ($P < 0.05$) linearly related to tryptophan level (table 1). The intersect with a daily nitrogen balance of 1 g occurred at 0.012% (5 mg/day/kg$^{3/4}$) dietary tryptophan, which we regard as the maintenance need for this amino acid. The reasoning behind the selection of 1 g/day nitrogen retention for estimating the maintenance requirement has already been discussed (2). The intersect with a nitrogen balance of zero indicated the tryptophan requirement for nitrogen equilibrium to be 0.004% (2 mg/day/kg$^{3/4}$).

Total urinary nitrogen, as well as urea plus ammonia nitrogen, decreased linearly ($P < 0.05$) as dietary tryptophan increased. The percentage of total urinary nitrogen contributed by urea plus ammonia varied significantly ($P < 0.01$) with the individual gilt, the period and the tryptophan level. Creatinine nitrogen excretion was not influenced by level of tryptophan but was significantly ($P < 0.05$) influenced by gilts. However, when expressed as a percentage of total urinary nitrogen, creatinine increased linearly ($P < 0.05$) in response to increasing dietary levels of tryptophan.

Methionine. The results of this assay appear in table 2. Linear response to methionine in the absence of cystine was significant ($P < 0.01$). Maintenance required 0.059% (26 mg/day/kg$^{3/4}$), whereas nitrogen equilibrium required 0.028% (12 mg/day/kg$^{3/4}$) dietary methionine.

Differences due to period of collection were not statistically significant in either this or the tryptophan assay, suggesting that stage of estrous cycle had little if any effect on nitrogen retention. In both assays estrus occurred during the collection portion of period B, yet nitrogen utilization in this period was not different from that noted in periods A or C.

To estimate the replacement value of cystine for methionine, 3 observations

TABLE 1

Nitrogen metabolism of gilts fed graded levels of tryptophan

Tryptophan level	Period [2]	Gilt no.[3]	Daily N [1]				
			Urine			Feces	Balance
			Creatinine	Urea and NH_3	Total		
			g	g	g	g	g
0	A	1	1.39	6.69	9.48	1.25	−0.71
	B	3	1.48	6.38	9.35	1.41 [4]	−0.74
	C	2	1.27	6.28	8.52	1.65 [4]	−0.15
	Avg		1.38	6.45	9.12	1.44	−0.53
0.02	A	2	1.30	3.75	5.87	1.65	2.50
	B	1	1.54	3.05	6.08	1.25 [4]	2.69
	C	3	1.45	4.78	7.47	1.41 [4]	1.14
	Avg		1.43	3.86	6.47	1.44	2.11
0.04	A	3	1.51	4.93	7.35	1.41	1.26
	B	2	1.39	3.31	6.17	1.65 [4]	2.20
	C	1	1.45	3.25	5.66	1.25 [4]	3.11
	Avg		1.45	3.83	6.39	1.44	2.19

[1] Daily N intake was 10.02 g.
[2] Periods were 10 days in length with urine collections being made on the last 5 days of each period.
[3] Three crossbred gilts were used in a Latin square design.
[4] Values observed during period A.

TABLE 2

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

Methionine level	Period [3]	Gilt no.[4]	Daily N [2]				
			Urine			Feces	Balance
			Creatinine	Urea and NH_3	Total		
%			g	g	g	g	g
0	A	4	1.51	6.76	9.43	1.65	−1.06
	B	6	1.48	6.52	9.31	1.43 [5]	−0.72
	C	5	1.50	7.18	9.61	1.53 [5]	−1.12
	Avg		1.50	6.82	9.45	1.54	−0.97
0.05	A	5	1.48	5.96	8.10	1.53	0.39
	B	4	1.52	4.19	7.35	1.65 [5]	1.02
	C	6	1.49	5.22	7.59	1.43 [5]	1.00
	Avg		1.50	5.12	7.68	1.54	0.80
0.10	A	6	1.48	4.31	6.81	1.43	1.78
	B	5	1.51	3.97	6.87	1.53 [5]	1.62
	C	4	1.51	3.32	6.02	1.65 [5]	2.35
	Avg		1.50	3.87	6.57	1.54	1.92

[1] Basal diet contained no cystine.
[2] Daily N intake was 10.02 g.
[3] Periods were 10 days in length with urine collections being made on the last 5 days of each period.
[4] Three crossbred gilts were used in a Latin square design.
[5] Values observed during period A.

were made with a diet containing 0.10% cystine but no methionine. The resulting average nitrogen balance was 0.94 g/day indicating that cystine at this level and for this length of time (10 days) can supply 94% of the maintenance need for sulfur amino acids. Perhaps there is a combination of these 2 amino acids which would result in 1 g of nitrogen retention at a level of sulfur amino acids less than the 0.059% methionine found to be required for maintenance.

A general observation has been that cystine has a higher replacement value for methionine for maintenance than for growth. Rose and Wixom (5) observed that about three-quarters of the methionine requirement of the adult rat could be supplied by cystine, whereas only about one-sixth could be so supplied in the diet of the growing rat. Becker et al. (6) suggested that cystine could supply approximately 40% of the total sulfur amino acid need for optimal growth of the young pig, which is in marked contrast with the estimate of 94% found in the present experiment for maintenance. The explanation for this apparent disparity may be that a substantial portion of the maintenance requirement of amino acids is for keratin synthesis, whereas keratin synthesis is a minor factor in the requirement for growth. Cystine is present in extremely high concentration in swine hair, being over one-fifth of the total protein (table 3). In contrast, methionine is present in very low concentration. This might explain why at maintenance a substantial portion of the total sulfur amino acid need is for cystine. Commenting on the importance of the amino acid composition of keratins in dictating the maintenance requirements of amino acids Mitchell (7) said: "The high cystine content of the proteins of these structures is outstanding in comparison with the soft tissues of the animal body, and, strange as it may seem, when the supply of cystine and methionine, either exogenous or endogenous, is deficient for the normal rate of synthesis of keratins, the keratinogenous tissues are able to raid the soft tissues, which are ordinarily considered the more essential for life, to secure the needed amounts of sulfur-containing amino acids."

It has been observed frequently that methionine, when added to a nitrogen-free

TABLE 3
Amino acid composition of swine hair [1,2,3]

	g/16 g N
Aspartic acid	6.23
Threonine	5.98
Serine	7.46
Proline	6.77
Glutamic acid	15.78
Glycine	3.35
Alanine	4.20
Valine	5.53
Cystine	21.99
Methionine	0.47
Isoleucine	3.17
Leucine	7.43
Tyrosine	2.38
Phenylalanine	2.09
Lysine	3.12
Histidine	1.01
Arginine	8.92

[1] Average of duplicate analyses of a pooled sample from 8 gilts.
[2] Crude protein (N × 6.25) was 91.75%.
[3] Determined by the method of Spackman et al. (14) utilizing a Beckman 120 B automatic amino acid analyzer.

diet, depresses the excretion of urinary nitrogen. Hence, when a nitrogen-free diet is fed over an extended period, the need of sulfur amino acids for maintenance and synthesis of keratin may stimulate tissue protein catabolism in an effort to provide needed methionine or cystine or both. Thus, supplementary methionine may do nothing more than depress the rate of tissue protein catabolism and thereby reduce urinary nitrogen excretion.

Urinary nitrogen derived from urea and ammonia, expressed either as grams per day or as a percentage of total urinary nitrogen, decreased linearly ($P < 0.01$) as methionine level increased. Also, both these criteria were significantly ($P < 0.05$) influenced by periods and by gilts. Urinary nitrogen derived from creatinine, expressed as grams per day, was uninfluenced by methionine level. However, when expressed as a percentage of total urinary nitrogen, creatinine nitrogen increased linearly ($P < 0.01$) as methionine level increased.

Creatinine excretion was not depressed by feeding a methionine-free diet for 10 days, despite the observed negative nitrogen balance, indicative of a decrease in lean body mass, and despite the known function of methionine as a methyl donor in creatine (hence, creatinine) synthesis. Perhaps a longer feeding period is necessary to manifest this effect. Fisher (8) reported that creatinine excretion of rats was depressed by a methionine-free diet if fed for at least 14 days.

Lysine. A significant ($P < 0.05$) linear response to graded levels of lysine occurred despite the fact that the lysine-void diet effected only a slightly negative nitrogen balance (table 4). The lysine requirement was found to be 0.058% of the diet (25 mg/day/kg$^{3/4}$) for maintenance, 0.014% of the diet (6 mg/day/kg$^{3/4}$) for nitrogen equilibrium.

The adult chicken does not require lysine for nitrogen equilibrium (9), and those species shown to have a lysine requirement need only a very small quantity. The adult rat requires only 3.6 mg/day/kg$^{3/4}$ dietary lysine (10). Lysine is present at a low concentration not only in swine hair (table 3), but in rat hair and chicken feathers as well (11). Perhaps as Leveille et al. (9) showed for chickens, a direct relation exists between the amino acid composition of keratoid tissues and the corresponding amino acid requirements for maintenance.

Urinary nitrogen derived from urea and ammonia decreased ($P < 0.05$) linearly as dietary lysine level increased, whereas that derived from creatinine was uninfluenced by the level of lysine fed. Expressed as a percentage of total urinary nitrogen, urea and ammonia decreased, whereas creatinine increased, the latter significantly ($P < 0.05$) linearly, as lysine intake increased. In this assay, as well as the other assays in which a partial fractionation of urinary nitrogen was performed, 80 to 90% of the total urinary nitrogen could be accounted for by these 3 components.

Glutamic acid (nonspecific amino nitrogen). The adequacy of the determined requirements was tested by feeding diets containing the essential amino acids at their calculated requirement levels for maintenance, but at graded levels of glutamic acid intake. Thus, no arginine, histidine or leucine was present in the diets fed in this assay. Due to an extremely slow rate of passage (accompanied by constipation) by all 6 gilts involved in this assay, quantitative fecal collection was impossible. Therefore, to facilitate calculation of nitrogen balance, an average fecal

TABLE 4

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

Lysine level	Period [3]	Daily N [2]				
		Urine			Feces [4]	Balance
		Creatinine	Urea and NH_3	Total		
%		g	g	g	g	g
0	A	1.34	6.24	8.51	1.41	−0.09
	B	1.33	7.20	8.70	1.65	−0.52
Avg		1.34	6.72	8.61	1.53	−0.31
0.072	A	1.44	4.08	6.66	1.43	1.74
	B	1.53	5.03	7.68	1.25	0.90
Avg		1.49	4.56	7.17	1.34	1.32
0.144	A	1.32	3.42	5.54	1.65	2.64
	B	1.42	3.21	5.51	1.53	2.79
Avg		1.37	3.32	5.53	1.59	2.72

[1] Six crossbred gilts which had been used in a previous assay.
[2] Daily N intake was 9.83 g.
[3] Periods were 10 days in length with urine collections being made on the last 5 days of each period.
[4] Values observed during previous assays with these gilts.

nitrogen excretion was calculated from all of the available observations in which the purified diet had been fed. A significant ($P < 0.05$) linear response to glutamic acid occurred (table 5). A maximal nitrogen balance of 0.79 g/day was obtained at the 3% level of dietary glutamic acid. This falls only slightly short of the predicted balance of 1 g/day, the adopted value taken to be commensurate with maintenance. It is possible, even likely, that the fecal value assumed in arriving at these balances was greater than the amount actually excreted by gilts in this assay. A general observation throughout the course of these experiments was that a smaller quantity of nitrogen is excreted via the feces when the passage rate is slow.

The indispensable-to-dispensable amino acid nitrogen (I/D) ratio of the 3% glutamic acid diet was only 0.168. According to the literature, a ratio such as this is highly unfavorable for growth. Stucki and Harper (12) reported that the ratio should be at least one for optimal performance of growing rats and two for optimal performance of growing chicks. On the other hand, Rose and Wixom (13) found that an I/D ratio considerably below one would permit optimal nitrogen utilization in adult men. From the results of the assay reported here it must be concluded that a low I/D ratio had no detrimental effect on nitrogen utilization. If an I/D ratio of one had been required, it is unlikely that nitrogen equilibrium could have been achieved because glutamic acid could have been only 0.5%, and total nitrogen content of the diet only 0.96%, far below the

TABLE 5

Nitrogen metabolism of gilts fed graded levels of glutamic acid [1,2]

Glutamic acid level		Daily N		
		Intake	Urine	Estimated balance [3]
%		g	g	g
1.0		4.27	3.41	−0.47
		4.27	3.44	−0.50
	Avg	4.27	3.43	−0.49
2.0		5.77	4.84	−0.40
		5.77	4.20	0.24
	Avg	5.77	4.52	−0.08
3.0		7.41	5.29	0.79
		7.41	5.30	0.78
	Avg	7.41	5.30	0.79

[1] All amino acids were present at their requirement levels for maintenance (1 g N retention/day).
[2] Six crossbred gilts.
[3] Extremely slow passage made quantitative fecal collection impossible. An average fecal nitrogen excretion (1.33 g/day) was calculated from all available observations in which the purified diet had been fed, and this value was used to calculate nitrogen balances.

level needed to replace the endogenous losses of nitrogen. Assuming a daily endogenous loss of 4.5 g, replacement would require at least 2% glutamic acid in this particular diet. Because the essential amino acid requirements for maintenance are so low, a low I/D ratio is absolutely essential to the establishment of maintenance nitrogen balance with a diet containing the essential amino acids at only their requirement levels.

A summary of the amino acid requirements of the nongravid gilt is presented in table 6. Nitrogen equilibrium requirements were calculated so as to facilitate a comparison with the requirements for nitrogen equilibrium of man and the adult rat as calculated by Nasset (10). Four amino acids (arginine, histidine, leucine and phenylalanine) were nonessential for nitrogen equilibrium of the gilt, in contrast with two (arginine and histidine) and one (arginine) found to be nonessential for man and the adult rat, respectively. Except for threonine, and lysine in the case of the adult rat, the remaining indispensable amino acids were required in lesser quantity per unit of metabolic body size by the gilt than by the rat or by man.

It is difficult to compare maintenance amino acid requirements among, and even within, species because of differences in approach to assessing the maintenance condition. As was the case here, and of necessity, small numbers of animals have been used under conditions not approaching the practical. Open to question in most of the maintenance work reported to date is the role amino acid turnover rates play in dictating amino acid requirements. Caution must be exercised in attempting to extrapolate requirements for maintenance, such as those reported herein, to an entire population.

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TABLE 6

Amino acid requirements for maintenance and for nitrogen equilibrium computed from nitrogen balance studies with the adult nongravid gilt [1]

Amino acid [2]	Starting mixture [3]	Maintenance requirement [4]	N equilibrium requirement [5]
		mg/day/kg$^{3/4}$	
Arginine	79	0	0
Histidine	35	0	0
Leucine	126	0	0
Phenylalanine [6]	44	21	0
Lysine	63	25	6
Threonine	52	39	24
Isoleucine	65	30	20
Valine	70	21	11
Tryptophan	17	5	2
Methionine [7]	44	26	12

[1] Average weight was 145 kg.
[2] L-Isomer.
[3] Based on composition of a 3% crude protein corn-soybean meal mixture (wt ratio of 4.4:1).
[4] That quantity required for a daily N retention of 1 g.
[5] That quantity required for N equilibrium.
[6] In the absence of tyrosine.
[7] In the absence of cystine.

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Oxidation of ^{14}C-labeled Carbohydrate, Fat and Amino Acid Substrates by Zinc-deficient Rats [1]

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ABSTRACT To assess the nature of the major metabolic defects resulting from zinc deficiency, the extent and pattern of oxidation to respiratory CO_2 of various ^{14}C-labeled substrates were studied in fasted zinc-deficient rats and fasted control rats. Oxidations of intraperitoneally injected tracer doses of ^{14}C-labeled glucose, acetate, palmitate and glutamate were essentially unaffected by zinc deficiency. Oxidation of large doses of glucose and acetate administered on a body surface area (weight$^{2/3}$) basis also were little affected by zinc deficiency. Energy metabolism appears not to be impaired in the zinc-deficient rat. Oxidation of tracer doses of ^{14}C-labeled leucine and lysine was significantly enhanced in zinc deficiency and, as shown for leucine, this increase was prevented by feeding a zinc-supplemented diet for 31 hours before the 17-hour fast. Also, oxidation of large doses of glutamate, leucine and lysine was enhanced in zinc-deficient rats, but it was shown with the large dose of lysine that about half of the increased oxidation was due to the difference in body weight. However, a portion of the increased oxidation of the large doses of lysine, and presumably of leucine, was due to zinc deficiency per se. These results suggest a defect in protein synthesis in the zinc-deficient rat.

Zinc is an essential component of several dehydrogenases involved in carbohydrate metabolism (1), and a deficiency of zinc affects carbohydrate utilization in microorganisms. A decrease in fructose-1, 6-diphosphate aldolase activity of *Aspergillus niger* in zinc deficiency (2, 3) may explain the observation that the zinc requirement for mycelial growth of this organism was 7 times higher when the carbon source was glucose or glycerol than when it was citric or gluconic acids (4). The activities of 2 tricarboxylic acid cycle enzymes, isocitrate dehydrogenase and fumarase, also were decreased in zinc deficiency in *A. niger* (3). Disturbances in the metabolism of carbohydrate or fat in zinc-deficient animals, however, have not been described. Protein synthesis is depressed in the zinc-deficient tomato plant (5) and free amino acids accumulate (6), but these observations have not been extended to the animal organism.

The work described in the present report was concerned with $^{14}CO_2$ formation from ^{14}C-labeled D-glucose, sodium acetate, sodium palmitate, L-lysine, L-leucine, and L-glutamic acid injected into control and zinc-deficient rats. It was hoped that the nature of the major metabolic defect in the zinc-deficient rat would be revealed.

METHODS AND MATERIALS

Male weanling rats of the Holtzman strain were obtained at 21 days of age and placed individually in stainless steel cages. Half of the rats were fed the low zinc diet (assayed 1.1 ppm zinc) (table 1) and the other half were fed this diet with zinc carbonate supplementation (assayed 16.1 ppm zinc). After 4 weeks the rats receiving the low zinc diet were judged to be zinc-deficient by the depressed growth, cracking paws, thinning hair coat, and red rough tails. Conjunctivitis also was noted in many of these rats. A similar eye condition has been described in zinc-deficient dogs (7).

The zinc-deficient rats weighed 80 to 100 g at 4 weeks, whereas the zinc-supplemented rats weighed 150 to 200 g. Because zinc deficiency decreased food intake and to some extent feed efficiency (i.e., body weight gain per gram of food consumed), comparison of zinc-deficient rats with pair-fed or weight-paired control

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TABLE 1

Composition of diet

	g/kg
Glucose monohydrate [1]	718.8
Casein hydrolysate [2]	180.0
L-Tryptophan [3]	2.2
Choline chloride [4]	1.5
Corn oil	50.0
Salt mix [5]	44.9
Vitamin mix [6]	2.5
α-Tocopherol [4]	0.1

[1] Cerelose 2001, Corn Products Company, New York.
[2] Acid-hydrolyzed, salt-free. General Biochemicals Inc., Chagrin Falls, Ohio.
[3] General Biochemicals Inc., Chagrin Falls, Ohio.
[4] Solution in ethyl alcohol.
[5] Contained: (in grams) $CaCO_3$, 12.30; $CaHPO_4$, 0.267; KH_2PO_4, 17.062; NaCl, 12.50; $MgSO_4$, 2.425; $Fe_2(SO_4)_3 \cdot xH_2O$ (78.8% $Fe_2(SO_4)_3$), 0.225; and (in milligrams) $CuSO_4$, 58; KI, 0.249; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.175; 35.56 mg $ZnCO_3$ were added to make zinc-supplemented diet.
[6] Contained: (in milligrams) thiamine·HCl, 5; riboflavin, 5; niacin, 25; Ca pantothenate, 20; pyridoxine·HCl, 2.5; folic acid, 0.02; menadione, 0.5; biotin, 0.1; cyanocobalamin, 0.02; inositol, 100; and ascorbic acid, 50. Vitamins A (4000 IU/kg diet) and D_2 (2000 IU/kg diet) added as water-dispersable form in gelatin. Sucrose carrier.

TABLE 2

Amounts of substrates administered

Substrate	Substrate/100 cm² of estimated surface area [1]	
	Tracer dosage	Carrier dosage [2]
	μmoles	μmoles
D-Glucose	0.080	277
Na acetate	0.032	150
Na palmitate	0.044	0.044 [3]
L-Glutamic acid	0.016	60
L-Leucine	0.0022	60
L-Lysine	0.0016	60

[1] Containing in each case from 0.4 to 1.0 μCi ^{14}C/100 cm² surface area.
[2] Solutions of the carrier doses were isotonic and of neutral pH.
[3] The carrier dosage of palmitate was administered by intubation of about 0.5 ml of a 35% tripalmitin emulsion/100 cm² 2 hours before intraperitoneal injection of tracer dose.

rats was suggested. However, the altered feeding pattern (i.e., once per day) of pair-fed or weight-paired rats compared with that (i.e., ad libitum) of zinc-deficient rats or unrestricted control rats would be expected to alter the fate of labeled substrates more than would voluntary restriction in total food intake. Thus, pair-feeding was not used. To eliminate or at least minimize any effect of the difference in food intake, food was removed on the afternoon of the day before administration of the labeled compound and the rats were fasted 17 hours (overnight) before injection.

After fasting, each rat was injected intraperitoneally with the labeled substrate and was placed immediately in a respiration chamber constructed from a transversely bisected 2-liter Pyrex bottle. CO_2-free air was pulled through the bottle by slight suction. Respiratory CO_2 was collected by bubbling the air leaving the bottle through ethanolamine-ethylene glycol monomethyl ether (1:2) (8) above a glass fritted disc in a tall Pyrex column. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (8).

The following radiochemicals were used: D-glucose-UL-^{14}C (6.3 mCi/mmole); sodium acetate-1-^{14}C (16 mCi/mmole); palmitic-1-^{14}C acid (4.43 mCi/mmole); L-glutamic acid-UL-^{14}C (25 mCi/mmole); L-leucine-UL-^{14}C (180 mCi/mmole) [3]; and L-lysine-UL-^{14}C (248 mCi/mmole).[4]

The rats were weighed immediately prior to injection and body surface in square centimeters was estimated as $10W^{2/3}$, where W is body weight in grams (9). Since metabolic rate expressed on this basis was reported to be reasonably constant for animals of different sizes, the dosages of labeled compound were equalized per unit of estimated surface area. Two types of dosages were given. The first was a tracer dose, containing a small amount of material of high specific radioactivity. The second type of dose contained a large amount of non-radioactive substrate (carrier dose) together with a small amount of radioactive substrate, to test for a limitation in the enzyme systems involved in oxidation of the substrate.

The actual amounts of substrate injected into the rats are listed in table 2. The carrier solutions were isotonic or hypotonic. It was impossible to prepare a carrier palmitate solution because of the low solubility of sodium palmitate and palmitic acid; hence 2 hours before injection of palmitic-1-^{14}C acid, sodium salt, as the albumin complex, half of the deficient rats of average weight 96 g received 1.0 ml of a tripalmitin emulsion by intubation under light ether anesthesia and half of the zinc-supplemented rats of average weight 202 g received 1.8 ml of emulsion in the

[3] All obtained from Volk Radiochemical Company, Skokie, Illinois.
[4] Obtained from New England Nuclear Corporation, Boston.

same manner. The aqueous emulsion contained 35% tripalmitin and 10% Tween 20.[5]

RESULTS

The percentages of the dose of each substrate oxidized in 4 subsequent one-hour periods by zinc-deficient and control rats are listed in table 3. Data for both the tracer dosage and the carrier dosage of each substrate are included.

Oxidation of the tracer dose of glucose was not altered by zinc deficiency. Similar percentages of the carrier dose were also oxidized in 4 hours by control and zinc-deficient rats despite the large differences in weight between the 2 groups. Only slight differences were found in the percentages oxidized in each hour interval

[5] Polyoxyalkylene derivative of sorbitan monolaurate, Atlas Powder Company, Wilmington, Delaware.

TABLE 3

Oxidation of ^{14}C-labeled substrates to CO_2 by fasted zinc-deficient and fasted control rats

Substrate	Collection interval [1]	Amount of dose oxidized in each interval			
		Tracer dosage		Carrier dosage	
		Without Zn	With Zn	Without Zn	With Zn
	hours	%	%	%	%
Glucose	0–1	15.2 ± 1.8 [2]	15.0 ± 0.5	8.0 ± 0.6	7.9 ± 0.6
	1–2	16.4 ± 0.6	16.4 ± 0.9	10.5 ± 0.4	11.8 ± 0.8
	2–3	9.5 ± 0.2	9.6 ± 0.7	9.0 ± 0.4	10.0 ± 0.6
	3–4	5.5 ± 0.4	5.1 ± 0.5	6.9 ± 0.2	6.2 ± 0.4
Total	0–4	46.8 ± 2.7	46.1 ± 1.8	34.3 ± 1.1	35.9 ± 1.5
Acetate	0–1	63.8 ± 0.5	60.9 ± 1.3	53.7 ± 2.3	50.0 ± 0.6
	1–2	8.7 ± 0.1	11.5 ± 0.6	19.6 ± 2.0	22.5 ± 0.6
	2–3	3.6 ± 0.1	3.4 ± 0.2	6.1 ± 0.6	6.4 ± 0.2
	3–4	2.0 ± 0.1	1.8 ± 0.1	2.6 ± 0.2	2.5 ± 0.1
Total	0–4	78.2 ± 0.6	77.6 ± 0.8	82.1 ± 1.3	81.4 ± 0.6
Palmitate	0–1	25.1 ± 2.7	26.6 ± 1.1	25.3 ± 3.1	27.0 ± 0.3
	1–2	12.3 ± 0.5	13.1 ± 0.7	15.4 ± 0.8	12.7 ± 0.3
	2–3	6.5 ± 0.2	5.0 ± 0.3	6.3 ± 0.4	5.4 ± 0.2
	3–4	4.1 ± 0.4	2.9 ± 0.1	3.6 ± 0.1	2.7 ± 0.1
Total	0–4	48.1 ± 2.2	47.6 ± 1.2	50.6 ± 1.9	47.8 ± 1.1
Glutamate	0–1	34.9 ± 1.1	34.8 ± 0.4	30.8 ± 1.2	25.4 ± 1.2
	1–2	13.5 ± 1.3	12.8 ± 0.2	14.6 ± 0.7	15.5 ± 0.7
	2–3	6.8 ± 0.9	5.4 ± 0.1	7.3 ± 0.3	7.8 ± 0.1
	3–4	3.4 ± 0.1	2.9 ± 0.1	4.2 ± 0.4	4.0 ± 0.2
Total	0–4	58.3 ± 1.2	55.9 ± 0.5	56.8 ± 0.8	52.7 ± 0.3
Leucine	0–1	11.0 ± 1.4	4.8 ± 0.3	24.3 ± 2.2	15.0 ± 0.6
	1–2	3.4 ± 0.4	2.3 ± 0.1	14.1 ± 0.7	12.8 ± 0.4
	2–3	1.8 ± 0.3	1.3 ± 0.1	6.0 ± 0.7	5.1 ± 0.3
	3–4	1.1 ± 0.1	0.8 ± 0.1	2.9 ± 0.2	2.1 ± 0.2
Total	0–4	17.4 ± 2.2	9.2 ± 0.5	47.4 ± 0.8	34.7 ± 0.4
Lysine	0–1	7.1 ± 0.7	5.6 ± 0.5	11.2 ± 1.2	5.7 ± 0.1
	1–2	3.8 ± 0.4	3.1 ± 0.1	8.3 ± 0.9	4.9 ± 0.4
	2–3	2.6 ± 0.3	2.0 ± 0.2	4.6 ± 0.4	3.0 ± 0.2
	3–4	1.8 ± 0.1	1.4 ± 0.1	2.9 ± 0.3	1.9 ± 0.1
Total	0–4	15.4 ± 0.8	12.1 ± 0.7	26.9 ± 2.6	15.5 ± 0.5

[1] Time after isotope injection.
[2] Mean ± SE. Four rats/group except 5 rats in glucose group without Zn.

after carrier dose administration. The delay in the peak oxidation of the carrier dosage relative to the tracer dosage (apparent when excretion of $^{14}CO_2$ was expressed for 20-minute intervals) was probably due to delayed absorption of the much greater amount of substrate and to the increased pool size.

By isotope dilution it was calculated that 5.9% of the CO_2 expired by the zinc-deficient rats and 5.3% of the CO_2 of the control rats came from the administered carrier glucose. The slightly higher percentages of the dose oxidized in control rats despite the lower percentage of the expired CO_2 from the administered carrier glucose is explained by the slightly higher rate of respiratory CO_2 excretion per unit of estimated body surface area in the larger control rats. The surface area basis of dose administration apparently was not ideal, but, since the error introduced by this non-ideality was not overcome by calculating a different function of body weight such as $W^{3/4}$ (9), the $W^{2/3}$ basis was used in subsequent trials.

About 80% of both the carrier and tracer dosages of acetate was oxidized in 4 hours by the control and zinc-deficient rats. However, with both substrate levels, the zinc-deficient rats oxidized a greater amount in the first hour and a smaller amount in the second hour than the zinc-supplemented rats. These observations indicate that the fractional turnover rate of acetate was somewhat faster in the zinc-deficient rat.

About half of the palmitate-^{14}C dose was oxidized to CO_2 in 4 hours by both control and zinc-deficient rats, whether they received the tripalmitin emulsion or not. Although the percentage of the dose oxidized in the first hour was greater in zinc-supplemented rats, the difference was small and variation within treatments was much greater than among treatments.

The percentage of the glutamate tracer dose oxidized to CO_2 in 4 hours by zinc-deficient rats was slightly higher than the percentage oxidized by control rats, but the 4% increase was not statistically significant. However, the carrier glutamate dose was oxidized to a significantly greater extent ($P < 0.05$) in 4 hours by zinc-deficient rats than by controls. This higher percentage of the carrier dose oxidized by zinc-deficient rats was entirely due to increased oxidation in the first hour.

Significantly higher ($P < 0.01$) percentages of both the tracer and carrier dosages of leucine were oxidized in 4 hours by zinc-deficient rats. Almost twice as much of the tracer dose was oxidized by deficient rats as by controls, and the deficient rats oxidized 37% more of the carrier leucine dose than controls. Most of the difference in oxidation of both dosages of leucine occurred in the first hour.

To determine whether the difference in leucine oxidation between the zinc-deficient and control rats was due to zinc deficiency directly or to the smaller size of the deficient animals, 2 groups of zinc-deficient rats were given the zinc-supplemented diet before leucine administration: one group for 31 hours before the 17-hour fast and the other for 7 hours before the start of the fast. The percentage of the tracer leucine dose oxidized in 4 hours was measured (table 4). The zinc-deficient rats again oxidized almost twice as much leucine as did the control rats. Rats fed the zinc-supplemented diet for 7 hours before food was removed had a slightly lowered oxidation, whereas rats fed this diet for 31 hours oxidized significantly less ($P < 0.05$) leucine than did zinc-deficient rats and compared closely in leucine oxidation with rats that were continuously zinc-supplemented and that weighed almost twice as much. Thus the increased oxidation of tracer leucine by zinc-deficient rats was reversible and had little dependence on animal size.

TABLE 4

Oxidation of a tracer dose of L-*leucine-*^{14}C *by control and zinc-deficient rats, and rats recovering from zinc deficiency*

Treatment	Amt of dose oxidized in 4 hr	No. rats	Body wt
	%		g
Without Zn	$21.0 \pm 1.5^{\alpha}$ [2]	4	104
With Zn, 7 hr [3]	$17.6 \pm 1.6^{\alpha,\beta}$	5	100
With Zn, 31 hr [3]	$14.4 \pm 1.1^{\beta,\gamma}$	5	100
With Zn	$11.8 \pm 0.6^{\gamma}$	4	176

[1] Mean ± SE.

[2] Means followed by superscripts containing the same Greek letter do not differ significantly ($P > 0.05$) as determined by a multiple range test (14).

[3] Zinc-deficient rats were fed the zinc-supplemented diet for 7 and 31 hours prior to the 17-hour fast which preceded leucine administration (at 24 and 48 hours before leucine injection).

The 4-hour oxidation of tracer lysine by zinc-deficient rats (table 3) was increased 25% over the control level ($P < 0.01$). Whereas most of the difference between the control and zinc-deficient rats in the percentage of the tracer leucine dose oxidized in 4 hours occurred in the first hour, the percentage of tracer lysine oxidized by the zinc-deficient rats was uniformly higher in all 4 one-hour intervals. Oxidation of the carrier dosage by zinc-deficient rats was increased over that by controls more with lysine than with glutamate or leucine. The percentage of the dose oxidized by the deficient rats was 74% greater than the percentage oxidized by the control rats. Again, unlike leucine, the increased oxidation of the carrier dosage of lysine persisted throughout all 4 one-hour intervals.

The effect of zinc administration to zinc-deficient rats on oxidation of the carrier dosage of lysine was determined. Zinc-deficient rats were injected with 100 μg of zinc as the glycine complex (10), at various times before administration of lysine. This amount of zinc is approximately the daily requirement of a 100-g rat. Increased oxidation of a carrier lysine dose by zinc-deficient rats was confirmed (table 5). Some decrease in the percentage of the lysine carrier dose oxidized was observed when deficient rats received zinc injections 24 and 48 hours before the start of the 17-hour fast.

To determine whether the body weight difference between control and zinc-deficient rats affected the oxidation of the carrier dosage of lysine, 3 groups of male Holtzman rats weighing 50, 100, and 150 g were fed the zinc-supplemented diet for one week. After fasting, the rats were injected with the carrier lysine dose on an estimated surface area basis. The percentage of the dose oxidized in 4 hours is plotted against body weight in figure 1. There was a highly significant ($P < 0.01$) negative correlation between body weight and carrier lysine oxidation when the dose was administered on the estimated body surface area basis. This could account in part for the higher oxidation of the carrier dosage of lysine in smaller, zinc-deficient rats. A similar highly significant ($P < 0.01$) negative correlation between body weight and percentage of a carrier lysine dose oxidized in 4 hours was observed when the dose was administered on a body weight basis (60 μmoles/50 g body weight). Therefore, with carrier dosages of the amino acids differences in body weight accounted for some, but not all, of the difference in extent of amino acid oxidation by zinc-deficient as compared with control rats,

TABLE 5

Oxidation of the carrier dose of L-lysine-^{14}C by control and zinc-deficient rats, and rats recovering from zinc deficiency

Treatment	Amt of dose oxidized in 4 hr	No. rats	Body wt
	% [1]		g
Without Zn	25.5 ± 1.3 $^{\alpha}$ [2]	4	95
With Zn, 17 hr [3]	25.6 ± 3.4 $^{\alpha}$	3	95
With Zn, 24 hr [3]	22.3 ± 1.5 $^{\alpha,\beta}$	3	89
With Zn, 48 hr [3]	21.7 ± 1.6 $^{\alpha,\beta}$	3	104
With Zn	17.8 ± 0.4 $^{\beta}$	3	158

[1] Mean ± SE.
[2] Means followed by superscripts containing the same Greek letter do not differ significantly ($P > 0.05$) as determined by a multiple range test (14).
[3] Zinc (100 μg as the glycine complex) injected intraperitoneally into zinc-deficient rats 17, 24, and 48 hours before lysine injection. All rats fasted 17 hours before lysine administration.

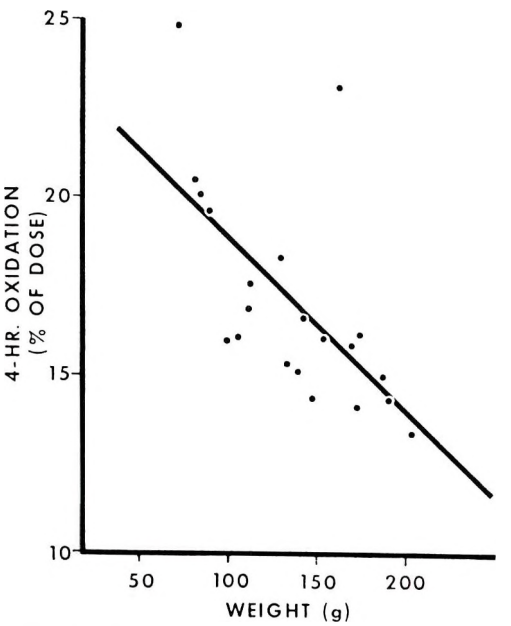

Fig. 1 Correlation of body weight and the percentage of a carrier dosage of lysine-^{14}C oxidized in 4 hours by fasted, control rats. The dose was injected on an estimated surface area basis.

but this was not the case for the tracer dosages.

DISCUSSION

The percentages of labeled glucose, acetate, and palmitate oxidized to respiratory CO_2 by rats were not affected by zinc deficiency, whether the substrates were administered in tracer quantities or in substantial (carrier) amounts. The fractional turnover rate of acetate appeared to be increased slightly in zinc-deficient rats, but the significance and cause of this alteration have not been demonstrated. The data indicate that little or no impairment in oxidative metabolism of carbohydrate or fat occurs in the rat due to zinc deficiency and that the effects on carbohydrate metabolism demonstrated in zinc-deficient microorganisms are probably not manifest in rats. This is not too unexpected since little decrease in soft-tissue zinc concentrations results in animals from dietary zinc deficiency. It also suggests that decreases in some zinc-containing nicotinamide adenine dinucleotide-dependent dehydrogenases in the tissues of zinc-deficient rats probably are not substantial.

The absence of decreased glutamate oxidation in the zinc-deficient rat probably indicates no substantial decrease in glutamic dehydrogenase, a known zinc metalloenzyme (11).

Contrary to the observations with glucose, acetate, palmitate, and glutamate, the oxidation of tracer doses of lysine and leucine was significantly increased in zinc-deficient rats. This effect was not related to the difference in body weight caused by zinc deficiency, because feeding the zinc-supplemented diet to deficient rats for 31 hours before the 17-hour fast reduced the oxidation of leucine to the control level. Thus the increased amino acid catabolism observed in zinc deficiency was readily reversible.

Increased oxidation of labeled amino acids in vitamin E-deficient rabbits has been reported (12, 13). After glycine-1-^{14}C injection the respiratory CO_2 of vitamin E-deficient rabbits had a higher specific activity than that of control animals (12, 13). The percentages of tracer doses of glycine, leucine, and lysine oxidized to CO_2 by vitamin E-deficient rabbits also were increased (13). These increases were attributed to vitamin E deficiency rather than to the state of inanition in the deficient animals since fasting did not alter leucine oxidation as much as did vitamin E deficiency, and because the oxidation of 3 non-amino acid substrates, glucose, acetate, and palmitate, were not altered significantly.

Similar results are reported here. However, the oxidation of the nonessential amino acid glycine was increased in vitamin E deficiency, whereas, in the present study, the oxidation of a tracer dose of the nonessential amino acid glutamic acid, was unaffected by zinc deficiency. The increased amino acid oxidation of vitamin E deficiency also was reversible, as injection of vitamin E into vitamin E-deficient rabbits 24 hours before glycine-1-^{14}C injection reduced the percentage of labeled glycine oxidized in 2 hours to the control level (13). Increased amino acid catabolism in vitamin E deficiency may be ascribed to an increased catabolism of protein in tissues such as muscle.

The results with tracer doses of labeled amino acids could be interpreted in a fairly straightforward manner; however, the results with the larger carrier dosages of the amino acids were complicated by an effect of animal size on the oxidation of these substrates. About half of the difference between the control and zinc-deficient rats in the extent to which they oxidized the large dose of lysine could be ascribed to the deficiency and half to the difference in body weight. Although not tested experimentally, the 8% higher oxidation of the carrier dose of glutamate by zinc-deficient rats was probably due almost entirely to the weight difference, but suggests again that the level of the zinc enzyme glutamic dehydrogenase is not limiting in the zinc-deficient rat. The oxidation of the carrier dose of leucine by zinc-deficient rats was much higher than that of controls and as in the case of lysine, can probably be attributed in part to zinc-deficiency and in part to body size.

The increased oxidation of leucine and lysine by fasted, zinc-deficient rats may be attributed either to a defect in protein synthesis or to accelerated protein cata-

bolism. Further research is needed to delineate the role of zinc in protein metabolism.

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Growth Depression and Pancreatic and Intestinal Changes in Rats Force-fed Amino Acid Diets Containing Soybean Trypsin Inhibitor [1,2]

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ABSTRACT This investigation was conducted with rats force-fed amino acid diets, compounded to simulate 14% soybean, in order to investigate whether soybean trypsin inhibitor (SBTI) could induce growth depression when food intake and protein availability were not contributing factors. Two groups were fed the basal diet with and without SBTI for 21 days. Two other groups were fed the same diets supplemented with methionine, threonine and valine. Two hours after the last feeding, pancreases were removed and the contents of the small intestine collected, lyophilized, then analyzed for protease activity and trichloroacetic acid-precipitable nitrogen. Amino acids were determined on the trichloroacetic acid (TCA) precipitate. Although food intakes were controlled, rats fed SBTI grew less, had enlarged pancreases, more intestinal protease and more TCA-insoluble nitrogen than did controls. Analysis of the TCA-insoluble material showed it to contain more essential amino acids and about 17 times more cystine than similar material from controls. Methionine, threonine and valine prevented the growth depression induced by SBTI, but did not prevent the intestinal changes. Thus, SBTI caused rat growth depression unrelated to food intake or availability of dietary amino acids. The results indicate that the mechanism of this effect appears to be due to a loss of the critical amino acids, methionine (via cystine), threonine and valine, caused by the SBTI's ability to stimulate the pancreas to discharge excessive quantities of endogenous protein into the intestinal tract. Although much of this endogenous nitrogen may be reabsorbed, bacterial degradation, especially of those amino acids most limiting for growth, would prevent their normal re-utilization in structural protein.

The demonstration by Ham et al. (1, 2) of a substance from raw soybean that inhibited intestinal proteolysis in chicks, and the ultimate isolation of a crystalline trypsin inhibitor from raw soybeans (3–5) have stimulated numerous investigations of the role trypsin inhibitors play in depressing the nutritional value of certain plant proteins. Unfortunately, because of different experimental designs and differences in interpretation of results, the relationship of trypsin inhibitor to growth depression in rats and chicks has not been satisfactorily clarified.

Recent studies in chicks and rats, however, have shifted the emphasis from amino acid availability and inhibition of intestinal proteolysis by soybean trypsin inhibitor (SBTI) toward the possibility of a much more direct effect on pancreatic function. The earliest report that raw soybean had physiological effects on the pancreas was made by Chernick et al. (6) who noted that chicks fed raw soybean in their diets had enlarged pancreases containing exceptionally high concentrations of trypsinogen. Subsequent studies with rats (7, 8) demonstrated that a single feeding of crystalline soybean or lima bean trypsin inhibitor or of an amorphous, ovomucoid trypsin inhibitor resulted in an exaggerated secretion of pancreatic protease, lipase, and amylase into the small intestine. Lyman (9) and Booth et al. (10) suggested that trypsin inhibitors might produce growth-retarding effects through stimulating increased enzyme secretion, with some of the endogenous nitrogen being incompletely reabsorbed and ultimately lost to the animal. In an effort to test this hypothesis, Haines and Lyman (11) fed

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rats heated soybean diets supplemented with a trypsin inhibitor concentrate, and demonstrated that over a 3-week interval the animals exhibited enlarged pancreases, did not grow as well as the pair-fed controls, and had higher intestinal nitrogen and protease activities. Rats fed raw soybean diets having the same trypsin inhibitor activity showed similar changes in their pancreases and intestinal nitrogen and enzymes, but their growth retardation was much more severe. The results suggested that endogenous nitrogen loss could have contributed to the growth depression, but that other factors in raw soybean were also influencing the results. Borchers (12, 13) has presented evidence which indicates that the metabolism of methionine, threonine, and valine may be impaired by some factor present in raw soybean meal. Barnes et al. (14) and Kwong and Barnes (15) also demonstrated that a factor in unheated soybean appears to interfere with the utilization or metabolism of methionine and cystine. Later reports by this group have indicated, however, that the factor responsible for the metabolic disturbance is probably SBTI (16).

The present study was conducted to establish whether SBTI caused growth depression in rats when food intake and protein availability were not factors, and, if so, whether the results were adequately explained by stimulated secretion of endogenous nitrogen.

MATERIALS AND METHODS

Animals and diets. Male rats of the Long-Evans strain, initially weighing about 60 g, were housed in separate metabolic cages, with constant access to water. They were tube-fed twice daily for 21 days, and weighed each morning prior to feeding.

A basal diet was prepared with proportions of purified amino acids that simulated those of amino acids in 14% soybean protein (17). (Composition of the diets is shown in table 1.) Sufficient SBTI concentrate was added to the diet to provide trypsin inhibitor activity equivalent to that of a diet containing 14% raw soybean protein. Control rats were fed the same diet except that trypsin inhibitor was replaced by an equivalent amount of inactive soybean protein. Thus, all diets contained approximately 1.0% intact protein in addition to the amino acids. In the second experiment, supplements of 0.6% DL-methionine, 0.6% DL-threonine and 0.5% DL-valine were added to the amino acid mix of both the control and experimental diets.

Caloric and nitrogen intakes of each group of rats were maintained at nearly equivalent levels by force-feeding a homogenate of the diet twice daily. Initially, 5 or 6 g of diet were fed, with daily increases until about 11 to 12 g (dry-weight equivalent) per rat per day were being fed. Details of the force-feeding procedures have been described elsewhere (18).

Soybean trypsin inhibitor concentrate was prepared from raw soybeans as described by Lyman and Wilcox (19). The activity of the preparation was about one-fourth that of crystalline trypsin inhibitor [3] when tested with crystalline trypsin by the method described by Borchers (20).

Sample collection and analytical determinations. During the last 4 days of the experiment, feces and urine were collected, and their total nitrogen was determined by a semimicro-Kjeldahl method which employed copper sulfate as a catalyst. On day 21 the animals were fed 5 ml of their respective diets, and 2 hours later they were exsanguinated by decapitation. Contents of the small intestine from the pyloric sphincter to the ileocecal junction were completely removed by pulling the opened intestine gently between a wet glass plate and a glass rod and gently washing into a container. Pancreatic tissue was carefully dissected from visible fat and mesentery tissue, under a microscope. The pancreas and intestinal contents were then frozen in solid CO_2 and lyophilized. Weighings and chemical analyses were made on this freeze-dried material. Pancreatic protease was activated by a 20-minute incubation with a 0.5% suspension of crude enterokinase preparation.[4] Pancreatic and intestinal protease activities were determined by the method of Anson (21) as modified for use with dried hemoglobin (22). Total pancreatic and intestinal nitrogen was measured by semimicro-Kjeldahl.

Intestinal protein was obtained by dissolving a weighed quantity of intestinal

[3] Worthington Biochemicals, Freehold, New Jersey.
[4] Viobin Corporation, Monticello, Illinois.

TABLE 1

Composition of diets

	With SBTI [1]	Without SBTI	Amino acid mix	
			Amino acid [2]	% in mix [3] equivalent to 14% protein
	% in diet	*% in diet*		
SBTI	1.4	—	L-arginine·HCl	1.16
Heated soybean protein	—	1.4	L-histidine	0.36
Amino acid mix	17.5	17.5	L-isoleucine	0.91
Fat-soluble vitamins [4]	1.0	1.0	DL-leucine	1.05
USP salts XIV [5]	4.0	4.0	L-lysine·HCl	0.95
Vitamin mix [6]	1.0	1.0	DL-methionine	0.14
Cottonseed oil	9.0	9.0	DL-phenylalanine	0.70
Cellulose [5]	10.0	10.0	DL-threonine	0.55
Sucrose	55.9	55.9	DL-tryptophan	0.14
Choline chloride	0.15	0.15	DL-valine	0.77
			DL-alanine	0.50
			DL-aspartic acid	0.87
			L-cystine	0.08
			L-glutamic acid	2.73
			Glycine	0.57
			L-proline	0.35
			DL-serine	0.97
			DL-tyrosine	0.48

[1] Soybean trypsin inhibitor.
[2] Calbiochemicals, Los Angeles.
[3] From Block and Weiss (17) to approximate the L-amino acids in 14% soybean protein.
[4] Cottonseed oil fortified to provide per kilogram of diet: vitamin A, 17,000 IU; vitamin D, 1000 IU; α-tocopheryl acetate, 66.6 mg; menadione, 5.0 mg.
[5] Nutritional Biochemicals Corporation, Cleveland.
[6] Sucrose mix to provide mg/kg diet: thiamine·HCl, 2.0; riboflavin, 3.0; pyridoxine, 2.5; pantothenic acid, 20.0; inositol, 100.0; biotin, 0.1; folic acid, 0.2; niacinamide, 10.0; vitamin B_{12} (as 0.1% triturate), 20.0.

TABLE 2

Effect of soybean trypsin inhibitor (SBTI) on growth of rats force-fed amino acid diets

Diet	No. of rats	Initial wt	21-day N intake	Total wt gain in 21 days
		g	*mg*	*g*
Basal	6	65 ± 3 [1]	4140 ± 40	42 ± 2
Basal + SBTI	8	68 ± 2	4047 ± 50	28 ± 4 [2]

[1] Mean ± SE.
[2] Significantly less than controls ($P < 0.01$).

contents in water and centrifuging out the insoluble material. Water-soluble protein was then precipitated with 10% trichloroacetic acid (TCA). The procedure was similar to that described by Chen et al. (23). Nitrogen was determined on the TCA precipitate and the water-soluble residue. The TCA precipitates were analyzed also for amino acid composition. A sample was digested in 6 N HCl for 10 hours, in sealed tubes, at 110°. The hydrolyzed material was then treated with 2,4 dinitro-1-fluorobenzene to form colored dinitrophenyl derivatives which were separated by 2-dimensional paper chromatography and quantitated colorimetrically (24).

Results were evaluated statistically by means of the *t* test as described by Snedecor (25). Differences between groups having a $P < 0.05$ were considered to be significant.

RESULTS

Nitrogen intake and 21-day weight gain of the rats fed the basal diet with and without SBTI are shown in table 2. The small difference between the 2 groups in terms of total nitrogen fed was the result

of feed consumption being equalized daily on a dry-weight basis, whereas nitrogen determinations on daily samples of the liquid diet were made at a later time. Even so, it is apparent that animals fed the SBTI concentrate did not gain as well as did the controls.

Table 3 shows the results of analyses of the intestinal contents from the same animals. Soybean trypsin inhibitor significantly increased total intestinal nitrogen and intestinal protease activity [5] ($P<0.01$). Measurement of the TCA-insoluble, TCA-soluble, and water-soluble nitrogen in the intestinal contents indicated that the higher nitrogen content in the intestine of rats fed SBTI can be attributed entirely to the TCA-insoluble fraction or to that portion which would represent principally intact protein. This proteinaceous material must have arisen as a result of feeding SBTI, and probably represents a combination of pancreatic enzyme secretions, trypsin-trypsin inhibitor complex and sloughings from the intestinal mucosa. Fecal nitrogen obtained during the last 4 days of the experiment was higher in the SBTI-fed rats, indicating that these animals continued to retain less nitrogen than did the controls throughout the experimental period.

If loss of critical amino acids through stimulation of pancreatic secretions contributed appreciably to the growth depression, supplementation with the appropriate amino acids should allow growth similar to that of the controls. To test this possibility, a second experiment was conducted in which the amino acids methionine, threonine and valine (reported by Borchers (12) to counteract growth depression in rats fed raw soybean diets) were added as supplements to the basic diets with and without the SBTI concentrates. Table 4 shows that while the unsupplemented rats fed SBTI

[5] Intestinal lipase and amylase activities were performed on a few animals and showed responses to the SBTI similar to that of the protease.

TABLE 3

Effect of soybean trypsin inhibitor (SBTI) on nitrogen and protease activity of intestinal contents and fecal nitrogen from rats force-fed amino acid diets

Diet	No. rats	Dry wt of intestinal contents	Total N in intestinal contents/ rat	Intestinal protease activity/ rat	TCA-insoluble N [1]	TCA-soluble N	Water-insoluble N [2]	Fecal N [3]
		mg	*mg*	*mEq tyr/10'*	*mg/rat*	*mg/rat*	*mg/rat*	*mg/rat/ day*
Basal	6	165 ± 20 [4]	3.5 ± 0.2	0.110 ± 0.006	0.58 ± 0.10	1.98 ± 0.26	0.98 ± 0.12	13 ± 1
Basal + SBTI	8	185 ± 15	5.8 ± 0.6 [5]	0.510 ± 0.017 [5]	2.34 ± 0.16 [5]	2.41 ± 0.38	1.20 ± 0.11	19 ± 1 [5]

[1] Trichloroacetic acid-insoluble nitrogen in intestinal contents.
[2] Insoluble nitrogenous material obtained by centrifugation after dissolving intestinal contents in water.
[3] Feces collected during last 4 days of experiment only.
[4] Mean ± SE.
[5] Significantly higher than controls ($P < 0.01$).

TABLE 4

Effect of soybean trypsin inhibitor (SBTI) on growth of rats force-fed amino acid diets with and without supplements of methionine, threonine and valine

Diet	No. of rats	Initial wt	Total N intake (21 days)	Total wt gain (21 days)
		g	*mg*	*g*
Unsupplemented				
Basal	4	65 ± 3 [1]	4350 ± 40	54 ± 1
Basal + SBTI	4	63 ± 4	4430 ± 30	42 ± 1 [2]
Supplemented [3]				
Basal	8	63 ± 4	4600 ± 30	55 ± 2
Basal + SBTI	8	63 ± 4	4500 ± 40	54 ± 2

[1] Mean ± SE.
[2] Significantly less than control ($P < 0.01$).
[3] Supplementation: 0.6% DL-methionine, 0.6% DL-threonine and 0.5% DL-valine.

TABLE 5

Effect of soybean trypsin inhibitor (SBTI) on the nitrogen and protease activity of intestinal contents and fecal nitrogen from rats force-fed amino acid diets with and without supplements of methionine, threonine and valine

Diet	No. rats	Dry wt of intestinal contents	Total N in intestinal contents/rat	Intestinal protease activity/rat	TCA-insoluble N [1]	TCA-soluble N	Water-insoluble N [2]	Fecal N
		mg	*mg*	*mEq tyr/10'*	*mg/rat*	*mg/rat*	*mg/rat*	*mg/rat/day*
Unsupplemented								
Basal	4	216 ± 10 [3]	4.8 ± 0.5	0.125 ± 0.008	0.54 ± 0.09	3.5 ± 0.3	0.73 ± 0.09	10.6 ± 0.9
Basal + SBTI	4	218 ± 25	7.4 ± 1.5 [4]	0.605 ± 0.049 [5]	2.85 ± 0.57 [5]	3.6 ± 0.2	0.94 ± 0.18	15.8 ± 1.1 [5]
Supplemented [6]								
Basal	8	247 ± 18	6.6 ± 0.7	0.177 ± 0.033	0.58 ± 0.07	4.9 ± 0.4	1.14 ± 0.17	12.1 ± 0.8
Basal + SBTI	8	244 ± 20	9.7 ± 1.3 [4]	0.487 ± 0.059 [5]	2.12 ± 0.48 [5]	6.3 ± 0.7	1.28 ± 0.23	13.5 ± 0.8

[1] Trichloroacetic acid-insoluble nitrogen in intestinal contents.
[2] Insoluble nitrogenous material obtained by centrifugation after dissolving intestinal contents in water.
[3] Mean ± SE.
[4] Significantly greater than control ($P < 0.05$).
[5] Significantly greater than control ($P < 0.01$).
[6] Supplementation: 0.6% DL-methionine, 0.6% DL-threonine and 0.5% DL-valine.

behaved like the animals of the previous experiment, i.e., failed to grow as well as the controls, the rats given the supplementary amino acids showed no growth depression when SBTI was included in the diet. The intake of nitrogen was raised somewhat in these animals by feeding more diet daily; thus 21-day weight gain was higher for these animals than for those in the first experiment (table 2). The higher intestinal TCA-insoluble nitrogen and protease activity (table 5) occurred regardless of the amino acid supplementation so that the influence of SBTI on the pancreatic secretion was not affected by the additional amino acids even though they restored growth to equal that of the controls. Fecal nitrogen excretion was not increased, however, when SBTI was fed to the supplemented animals. Pancreatic hypertrophy was also quite evident in these animals (table 6); rats fed SBTI had pancreases (dry weight) about twice the size of control animals. The increased size of the organ was also reflected in a greatly increased nitrogen content, which existed despite the fact that these animals had been stimulated and were secreting enzymes at the time they were killed. Judging from the reduced proteolytic enzyme activity in the SBTI-stimulated pancreases, secretion of active enzymes must have been taking place more rapidly than replacement.

Table 7 shows the amino acid analysis of the TCA-insoluble precipitate obtained from the small intestine of rats fed the basal diet with and without SBTI. In general, SBTI increased the level of the amino acids in this fraction to about 7 to 9 times that of the controls. Cystine secretion, however, was increased to about 17 times that of the controls, whereas histidine levels were raised about 25-fold. A few samples of intestinal contents from rats fed SBTI and supplemented with methionine, threonine and valine were also analyzed during these studies. Although cystine excretion in the group fed SBTI showed the disproportionate increase relative to the other amino acids, histidine excretion did not. Thus, the significance of the high excretion of histidine shown in table 7 is not clear. The SBTI apparently had no special effect on valine or threonine

TABLE 6

Effect of soybean trypsin inhibitor (SBTI) on the weight, nitrogen and protease content of the pancreas from rats force-fed amino acid diets with and without supplements of methionine, threonine and valine

Diet	No. of rats	Pancreas/100 g body wt		
		Dry wt	Nitrogen	Protease activity
		mg	*mg*	*(mEq tyr/10') × 10* [2]
Unsupplemented				
Basal	4	72 ± 7 [1]	7.1 ± 1.1	6.3 ± 0.8
Basal + SBTI	4	154 ± 2 [2]	18.1 ± 0.2 [2]	3.0 ± 0.4 [3]
Supplemented [4]				
Basal	8	82 ± 6	8.9 ± 0.7	9.4 ± 0.7
Basal + SBTI	8	195 ± 13 [2]	22.3 ± 1.5 [2]	5.7 ± 0.6 [3]

[1] Mean ± SE.
[2] Significantly greater than control ($P < 0.01$).
[3] Significantly less than control ($P < 0.05$).
[4] Supplementation: 0.6% DL-methionine, 0.6% DL-threonine and 0.5% DL-valine.

TABLE 7

Amino acid composition of trichloroacetic acid-insoluble protein from the intestinal contents of rats fed purified diets with or without soybean trypsin inhibitor (SBTI)

Essential amino acids [1]				Nonessential amino acids [1]			
	Basal	Basal + SBTI	Ratio [2]		Basal	Basal + SBTI	Ratio [2]
	μmoles/rat				*μmoles/rat*		
Histidine	14	354	25.3	alanine	116	987	8.5
Isoleucine + leucine	274	2082	7.6	aspartic acid + glutamic acid	181	1160	6.4
Lysine	137	1145	8.4	glycine	138	1245	9.1
Phenylalanine	55	357	6.5	proline	115	682	6.0
Threonine	91	685	7.5	serine	104	810	7.8
Valine	91	782	8.0	tyrosine	18	85	4.7
Cystine [3]	15	260	17.4				
Arginine	36	252	7.0				

[1] Values represent individual analyses from 4 rats in each group.
[2] Ratio = $\frac{\mu\text{moles amino acid with basal diet + SBTI}}{\mu\text{moles amino acid with basal diet}}$.
[3] Cystine is included in this group because of its close relation to the metabolism of methionine. Methionine was found only in trace quantities, although the technique was able to determine methionine in the hydrolyzed standards.

aside from the 7- to 8-fold increase in secretion of these amino acids into the small intestines.

DISCUSSION

The experiments described have shown that, under conditions in which food intake was controlled and maximal availability of dietary amino acids was achieved by tube-feeding a purified amino acid diet, SBTI concentrates produced a significant growth depression in rats. Moreover, supplementation of the SBTI diet with methionine, threonine, and valine completely prevented the growth-suppressing effect of the SBTI. This latter observation had been made previously (10, 12) with rats fed raw soybean diets. The SBTI preparation used in this experiment was therefore not, in itself, toxic to the animals, since they were forced to eat a certain amount of it rather than being allowed to decrease their food intake, as occurs under pair-feeding conditions.

Aside from a reduced growth rate, rats fed the SBTI concentrate appeared to differ from the controls only by having more intestinal protease activity, higher concentrations of TCA-insoluble nitrogen in the small intestine, and greatly enlarged pancreases. De Muelenaere (26) has reported a similar effect on the TCA-insoluble intestinal nitrogen in rats fed SBTI concentrates. In addition, he showed that the higher nitrogen was maintained through

the entire length of the small intestine and for a period of as long as 5 hours. Although growth was not reported, the author concluded that the higher intestinal nitrogen in rats fed SBTI resulted from excessive pancreatic secretions and slough-off from the intestinal mucosa. Thus, the results of De Muelenaere and those reported in the present study support the concept expressed previously by Lyman (9), Booth et al. (10) and Haines and Lyman (11), that SBTI acts as a growth depressant in the rat by stimulating the pancreas to secrete excessive quantities of digestive enzymes which are largely passed from the small intestine into the cecum and colon unabsorbed and ultimately lost to the animal. It is significant that during stimulation of active pancreatic secretion by SBTI, the animals maintained about 7 times the level of essential amino acids in the proteinaceous secretion than did the controls (table 7). However, the concentration of cystine was nearly 17 times that of the controls. Methionine is recognized as the most limiting amino acid in soybean protein (27). Since the diets used in our experiments were designed to have the proportions of the amino acids represented by 14% soybean protein, methionine levels would also be suboptimal. In the protein fraction recovered from the intestine of animals fed the SBTI, only traces of methionine were detectable, indicating that this amino acid per se was not being secreted excessively. The high output of cystine, however, would severely deplete methionine stores if this amino acid provided the major source of the cystine. Barnes and Kwong (16) have reported that in rats fed SBTI, the pancreas converted ^{35}S-labeled methionine into ^{35}S-labeled cystine more rapidly than did control animals. The present results show that rats fed SBTI secrete proteolytic enzyme (as well as amylo- and lipolytic enzymes) at 3 to 5 times the normal secretion (tables 3 and 5). Thus, our results are consistent with the interpretation of Barnes and Kwong that the increased conversion of methionine to cystine results from a need to supply cystine to meet the requirements of the newly synthesized and secreted enzyme proteins.

Supplementation of the diets with methionine, threonine and valine restored growth of the rats fed SBTI to the equal of the controls, yet the amino acids did not prevent the intestinal changes caused by the inhibitor. The beneficial effect of the supplementary amino acids appeared, therefore, to be replacement of growth-limiting essential amino acids. At the level of amino acids fed in this study, methionine is the most growth-limiting, with threonine and probably valine next (according to Borchers, (12)). Kwong and Barnes (15) demonstrated that cystine supplementation of raw soybean diets containing 10% protein did not restore growth completely to the level of that of rats fed heated soybean, whereas the same supplementation to diets equivalent to 25% protein did. These investigators felt that the results demonstrated a block in the utilization of cystine by some factor in raw soybean. In view of the present studies, however, it appears more likely that growth was restricted when the low protein diets were supplemented by cystine because of loss of the next growth-limiting amino acids (threonine or valine, or both) due to the exaggerated endogenous secretions. Thus, cystine alone could not provide for the increased losses of threonine or valine, and growth would therefore still be less than with the heated diets, in which excessive secretion of these essential amino acids would not occur.

The foregoing argument is valid only if a substantial part of the essential amino acids in the proteinaceous secretion is not available to the animal. Carroll et al. (28) had observed that rats fed raw soybean diets had more cystine in their intestinal contents than did controls, and suggested that bacterial destruction of the cystine could be a factor in the increased requirement for methionine. Lyman (9) demonstrated that from the small intestine to the colon, enzymatic activity of amylase, lipase and especially protease decreased sharply, suggesting also that hydrolysis and inactivation of the secreted enzyme protein were extensive after the material left the small intestine. Barnes et al. (29) demonstrated recently that penicillin prevented the growth depression in rats produced by feed-

ing raw soybean diets and increased the fecal excretion of cystine and proteolytic enzyme as well. When coprophagy was prevented by means of tail cups (30), penicillin was no longer effective. It was concluded that penicillin preserved a cystine-rich protein from which the cystine became available only after ingestion of the feces. In general, this interpretation is consistent with the concept that bacterial action degrades many of the amino acids in the endogenous secretions stimulated by SBTI to amines, mercaptans, organic sulfates and other nitrogenous products that are not utilizable by the rat for growth even though they may be absorbed. Penicillin and other antibiotics would provide their beneficial effects by altering the bacterial population so that destruction of cystine and other critical amino acids was minimized either at the level of the small intestine or during passage through the cecum or colon.

The enlargement of the pancreas in the rats fed SBTI (table 6) has also been reported previously for the rat (10, 11). Since other experiments had shown that crystalline SBTI (7) and crystalline lima bean trypsin inhibitor as well as ovomucoid (8) strongly stimulated pancreatic secretion when a single dose was fed to rats, we have felt that the pancreatic enlargement is a physiological response to the stimulation by the SBTI, a situation analogous, perhaps, to the hypertrophy induced in the thyroid or adrenal glands when they are hyperstimulated by thyroid-stimulating hormone or by adrenocorticotrophic hormone. Rackis et al. (31) have reported that crude soybean fractions, in which most SBTI activity had been removed, still produced pancreatic hypertrophy. As a consequence these investigators have postulated that raw soybean contains a "pancreatic hypertrophy factor" that acts independently of the trypsin inhibitor. Since the pancreatic enlargement produced by the soybean fractions was not very great when compared with the increase produced by raw soybean, final judgment on whether a "pancreatic hypertrophy factor" other than SBTI exists in raw soybean should be reserved until the results of Rackis and co-workers can be confirmed.

This study has demonstrated that SBTI produced growth depression in the rat that was unrelated to food intake, obvious toxicity or interference with availability of dietary amino acids. The mechanism by which SBTI causes this effect, and which appears to fit most closely recent experimental evidence, is that certain essential amino acids, namely methionine (in the form of cystine), threonine and valine, are lost to the animals as a result of the peculiar ability of SBTI to stimulate the pancreas to discharge exaggerated quantities of endogenous enzyme secretions into the intestinal tract, and from which the amino acids are not fully recovered by the animal. Borchers (12, 13, 32) has disagreed with this interpretation, however, and has presented evidence to indicate that a toxic factor in raw soybean interferes with the metabolism of methionine and, perhaps, threonine and valine as well (33, 34). In most of Borchers' studies, however, he has used raw soybean meal in the diet and it should be pointed out that the growth depression that we and others have observed in rats fed the SBTI concentrate was considerably less than that observed when the whole, raw soybean meal was fed (11, 35). Soybean is known to contain a number of toxic factors such as hemagglutinins, goitrogens, saponins, and various other substances,[6] as well as trypsin inhibitors (36). Although the present study has shown that SBTI may contribute to the growth depression induced by raw soybean, it is not the only factor involved. Since the present study was completed, Rackis (37) has reported that 10% and 14% casein diets supplemented with crystalline trypsin inhibitor depressed rat growth only to about 30 to 50% of that produced by raw soybean meal. The mechanisms by which the additional growth depression is induced by raw soybean meal are unknown and will probably remain controversial until all of the toxic components in raw soybean have been isolated and their relative biological effects, both individual and collective, have been established.

[6] A heat-labile substance in raw soybeans has been reported to interfere with fat absorbability in hens (38). On the possibility that decreased energy utilization might be a factor in our rat studies, a number of daily fecal samples from control and SBTI-fed rats were collected and analyzed for fat. However, no differences in fecal fat were observed.

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Effect of Dietary Linoleate on Chick Liver Fatty Acids: Dietary Linoleate Requirement [1,2]

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ABSTRACT Studies were conducted with chicks to determine changes in polyunsaturated fatty acids in liver lipids when the chicks were fed increasing amounts of dietary linoleate, and from this data the dietary requirement of the chick for linoleate was estimated. Increasing dietary amounts of linoleic acid fed to chicks as supplements of corn oil in a diet low in essential fatty acids (EFA) resulted in increased amounts of linoleate and arachidonate (18:2ω6 and 20:4ω6) and decreased amounts of eicosatrienoate (20:3ω9) in liver lipids, a characteristic of EFA deficiency. The ratio of trienoate to tetraenoate was plotted to estimate the requirement of the chick for linoleate, which was found to be 2.0% of dietary calories. The requirement estimated from the rate constant equation by computer methods was found to be the same. Equations were derived to allow an estimate of the dietary linoleate intake, based on fatty acid composition of the liver lipid.

The need of the growing chick for a dietary source of essential fatty acids (as linoleic or arachidonic acid) was investigated as early as 1950 (1), although the results and deficiency symptoms described may not have been due solely to an uncomplicated essential fatty acid (EFA) deciency (2). Other workers (2–8) have since concluded that either a source of fat or polyunsaturated fatty acids (PUFA) is necessary for the chick. However, none of these reports attempted a quantitative estimate of the requirement of the chick in terms of specific PUFA, although it has been stated that 3% corn oil appears to be adequate (4, 6). The adverse growth response of chicks as a criterion of EFA deficiency has proved variable. In some studies, it has been reported that an effect on growth appeared in 3 weeks (4, 5, 7). In other experiments no growth difference was noted in 6 weeks and very little up to 12 weeks (2, 3).

A biochemical lesion has been shown to be characteristic of EFA deficiency in rats (9), swine (10), dogs (11), guinea pigs (12), and humans (13) — that is, an increase of 5,8,11-eicosatrienoate (20:3ω9), accompanied by a decrease of linoleate (18:2ω6) and arachidonate (20:4ω6) in lipids of liver and other tissues. This biochemical abnormality has been reported to occur also in chicks that have been reared with a low fat diet (3, 5, 6, 8). The present studies were conducted to observe the changes in the content of linoleate, eicosatrienoate and arachidonate in liver lipid when chicks were fed varying amounts of dietary linoleic acid, and to determine more definitely the quantiative requirement of the chick for dietary linoleic acid from these data.

EXPERIMENTAL PROCEDURE

Male Mount Hope Queen Leghorn chicks were obtained from a local hatchery and reared in an electric battery brooder with raised wire floors. The chicks were watered, but not fed, for 48 hours. At 2 days the chicks were weighed individually, wing-banded and divided into groups. Since chicks from EFA-depleted hens were not available, the 2-day period without feed was used to expedite the depletion of the fatty acid reserves in the yolk sac because observations have been made showing appreciable stores of linoleate and arachidonate in the yolk lipids of incubating eggs (14). The chicks were fed the purified diets and water ad libitum, weighed weekly and killed at 7 weeks. The livers were excised, quick-frozen and stored at −20°.

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TABLE 1

Constant ingredients of basal diet [1]

	g/kg ration
Glucose [2]	570
Casein, vitamin-free	200
Fat [3]	50
Gelatin	80
Non-nutritive fiber [4]	20
Cholesterol	5
Methionine, hydroxy analogue, calcium	3.300
Chlortetracycline [5]	0.200
Choline chloride	2
Mineral mixture [6]	63
Vitamin mixture [7]	6.045
Vitamin A palmitate (250,000 IU/g)	0.100
Vitamin D_3 (200,000 IU/g)	0.005
d-α-Tocopheryl acetate	0.100
Ethoxyquin [8]	0.250

[1] Diet contained 3650 kcal/kg.
[2] Anhydrous Cerelose, Corn Products Company, Argo, Illinois.
[3] Supplied as a mixture of hydrogenated coconut oil and corn oil to give level of linoleic acid desired.
[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[5] Aureomycin, American Cyanamid Company, Princeton, New Jersey.
[6] Minerals provided the following (g/kg complete diet): $CaHPO_4$, 30.0; $NaHPO_4 \cdot 7H_2O$, 13.2; $CaCO_3$, 5.0; KCl, 7.0; NaCl, 4.0; $MgSO_4 \cdot 7H_2O$, 3.0; $FeSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.25; $ZnSO_4 \cdot H_2O$, 0.30; KIO_3, 0.01; $CuSO_4 \cdot 5H_2O$, 0.02.
[7] Vitamins in glucose provided the following (mg/kg complete diet): niacin, 100; Ca pantothenate, 20; thiamine·HCl, 8; riboflavin, 8; pyridoxine·HCl, 8; folacin, 3; vitamin K, 2; biotin, 0.30; and vitamin B_{12}, 0.02.
[8] 1, 2-Dihydro - 2, 2, 4 - trimethyl - 6 - ethoxyquinoline (Santoquin, Monsanto Company, St. Louis).

The basal diet is shown in table 1.[3] Graded levels of linoleate were fed by adding corn oil to the basal diet at the expense of the hydrogenated coconut oil, with amounts of dietary linoleate ranging from 0.016 to 5.80% of calories. Corn oil contains large amounts of linoleate and no arachidonate or any of the longer chain metabolites of linoleate. Two nutritional experiments were completed. Both experiments contained 10 groups of 12 chicks each. The length of experiment, methods of handling the feed and water, and methods of collecting samples were the same for both experiments.

Analyses of the lipid supplements were made by gas-liquid chromatography using a Beckman GC-2A instrument with a hydrogen flame detector. The column used was 1.83 m × 6.35 mm packed with 10% ethylene glycol succinate on Gas Chrom P, at 194°, with helium as the carrier gas. The peaks were identified by comparison with standards obtained from The Hormel Institute and NIH,[4] and by comparison of calculated equivalent chain lengths (15). The fatty acid composition of the lipids fed were (in % of total fatty acids): hydrogenated coconut oil; 8:0,[5] 5.9; 10:0, 5.3; 12:0, 44.1; 14:0, 20.9; 16:0, 9.3; 18:0, 11.8; 18:1ω9, 2.5; 18:2ω6, trace (less than 0.2); 20:0, trace. Corn oil; 12:0, trace; 14:0, trace; 16:0, 10.9; 16:1, 0.3; 17:0, trace; 17:1, trace; 18:0, 1.6; 18:1ω9, 27.2; 18:2ω6, 58.2; 18:3ω3, 1.5.

The liver samples were extracted with Folch reagent (chloroform-methanol, 2 to 1). The fatty acids were esterified with dry HCl-methanol. In experiment 1 the fatty acids were analyzed by alkaline isomerization (16), and in experiment 2 by gas-liquid chromatography as described above.

RESULTS AND DISCUSSION

The average chick weights at both 4 weeks and 7 weeks are shown in table 2. In experiment 1, at both 4 and 7 weeks, the weights for the chicks fed the 2 lowest levels of linoleate were lower than the weights of chicks fed greater amounts of linoleate. In the remainder of the groups there was wide variation with no significant differences. In experiment 2, the chicks showed little differences in weights at both 4 and 7 weeks, with the exception that the group fed the lowest level of linoleate grew at a slower rate than all the rest but one. These data substantiate those of workers who found growth effect a poor criterion for assessing EFA deficiency in chicks. The external appearance of the birds was not different, substantiating previous reports for chicks under 7 weeks of age (2, 6). The data do demonstrate that the weight responses shown were not due to a fat deficiency per se, as all groups were fed equal fat supplements, but reflected a specific need for linoleate as provided in the corn oil supplements.

[3] Materials used in these studies were generously supplied by: Distillation Products Industries, Rochester, New York; Abbott Laboratories, North Chicago, Illinois; Dawe's Laboratories, Inc., Chicago; Merck, Sharp and Dohme, Rahway, New Jersey; American Cyanamid Company, Princeton, New Jersey; the Monsanto Company, St. Louis; and DuPont de Nemours and Company, Wilmington, Delaware.
[4] Standards obtained through the courtesy of the NIH Lipid Distribution Program, Dr. W. N. Goldwater, Bethesda, Maryland.
[5] In polyunsaturated fatty acids, the number following the ω designates the position of the first double bond from the methyl (*omega*) end of the molecule.

TABLE 2

Average weights of chicks supplemented with dietary linoleate

Dietary linoleate	4 Weeks		7 Weeks	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
% of calories	*g*	*g*	*g*	*g*
0.016	131 ± 19 [1]	198 ± 9	248 ± 34	407 ± 27
0.13	152 ± 16		341 ± 28	
0.25	211 ± 14		414 ± 26	
0.50	200 ± 18	222 ± 8	423 ± 27	484 ± 17
0.75		223 ± 13		495 ± 24
1.00	198 ± 25	208 ± 13	405 ± 48	461 ± 21
1.25		196 ± 9		402 ± 23
1.50		222 ± 7		491 ± 19
1.75	170 ± 18	230 ± 9	330 ± 41	477 ± 15
2.00		227 ± 11		509 ± 23
2.30	232 ± 19	227 ± 8	440 ± 37	472 ± 19
2.50		220 ± 12		455 ± 23
2.90	233 ± 12		382 ± 24	
3.50	244 ± 25		377 ± 45	
5.80	229 ± 28		375 ± 48	

[1] SE of mean.

TABLE 3

Liver lipid fatty acids of chicks fed graded levels of dietary linoleic acid

Dietary linoleate	Exp. 1 — Isomerization data			Exp. 2 — GLC data		
	Dienoic	Trienoic	Tetraenoic	18:2ω6	20:3ω9	20:4ω6
% of calories	*% of total fatty acids*			*% of total fatty acids*		
0.016	1.1 ± 0.16 [1]	4.9 ± 0.40	1.2 ± 0.09	1.7 ± 0.05	8.6 ± 0.34	2.4 ± 0.11
0.13	2.0 ± 0.08	5.0 ± 0.30	1.7 ± 0.13			
0.25	3.0 ± 0.30	5.9 ± 0.42	2.7 ± 0.22			
0.50	4.1 ± 0.59	4.2 ± 0.31	3.9 ± 0.40	9.2 ± 0.44	5.4 ± 0.24	7.4 ± 0.34
0.75				11.1 ± 0.72	3.5 ± 0.33	8.3 ± 0.72
1.00	6.9 ± 0.40	3.5 ± 0.21	4.7 ± 0.40	11.8 ± 0.56	3.1 ± 0.13	9.4 ± 0.57
1.25				12.6 ± 0.49	2.7 ± 0.17	11.3 ± 0.93
1.50				16.3 ± 0.59	2.4 ± 0.15	16.5 ± 0.82
1.75	9.2 ± 0.63	3.0 ± 0.40	6.2 ± 0.29	13.6 ± 0.69	1.4 ± 0.09	14.5 ± 0.79
2.00				14.1 ± 0.34	1.6 ± 0.11	14.0 ± 0.56
2.30	6.9 ± 0.53	2.4 ± 0.19	4.7 ± 0.59	16.0 ± 0.59	1.4 ± 0.13	15.0 ± 1.01
2.50				17.5 ± 0.64	0.9 ± 0.06	19.1 ± 0.64
2.90	10.7 ± 0.62	1.6 ± 0.08	8.8 ± 0.63			
3.50	12.0 ± 0.91	1.0 ± 0.12	9.3 ± 0.62			
5.80	13.6 ± 0.49	1.1 ± 0.33	9.8 ± 0.71			

[1] SE of mean.

The characteristic biochemical lesion of tissue fatty acid imbalance in EFA deficiency was clearly demonstrated in both experiments as shown in table 3. The trienoic fatty acid (specifically 20:3ω9) was high when low linoleate was fed, and decreased markedly as the dietary linoleate increased. The liver dienoic and tetraenoic fatty acids (18:2ω6 and 20:4ω6) increased as the dietary linoleate increased. Between the 2 experiments, analyses of fatty acids from chicks fed equal linoleate intakes showed some differences. These could be due to differences in the chicks as well as differences in analytical methods. However, the conclusions drawn from the 2 experiments were the same.

An index useful in determining essential fatty acid requirement based on the tissue fatty acid composition is the ratio of trienoic to tetraenoic fatty acids, or more specifically, the ratio of 20:3ω9 to 20:4ω6 in the tissue lipids. This ratio has been used to determine the EFA requirement of rats (17), swine (18, 19), guinea pigs (12) and infants (20). The triene-to-

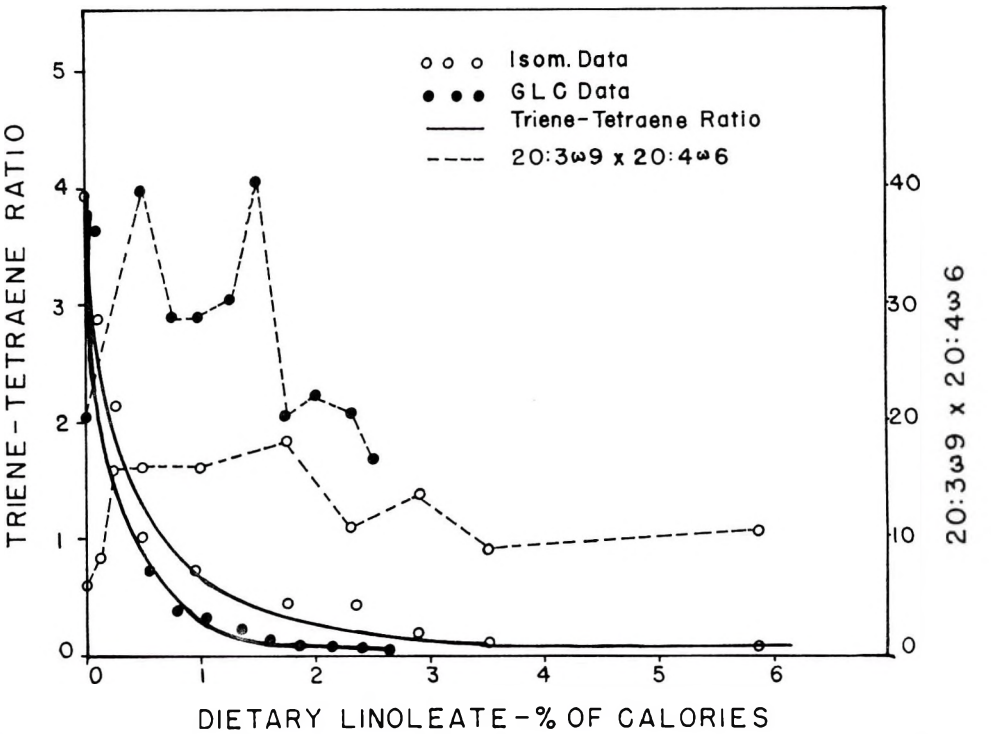

Fig. 1 Triene-to-tetraene ratio and triene × tetraene constant of liver fatty acids of chicks fed increasing amounts of dietary linoleate. In experiment 2 the ratio was calculated for % 20:3ω9/% 20:4ω6, and the constant was calculated for % 20:3ω9 × % 20:4ω6 in liver lipid.

tetraene ratios of the liver fatty acids of the chicks are shown in figure 1. In the 2 experiments the curves relating the triene-to-tetraene ratio to linoleate intake were almost superimposable. These curves were also similar to comparable curves derived from liver and other tissue lipids from other species tested (17–20). In both experiments the ratio decreased as dietary linoleate was increased in curves that were nearly identical. The linoleate requirement has been estimated from the points where the curves level off and the ratios fail to decrease rapidly with further increase in dietary linoleate. Both curves show this point to lie between 1 and 2% of dietary calories as linoleate. Since the gas-liquid chromatography data are more accurate than alkaline isomerization data, the curve for experiment 2 probably more nearly reflects the correct dietary linoleate requirement for the chick, and indicates that the requirement is not more than 2% of dietary calories. The value is very similar to that observed for rats (1%), swine (2%) and infants (1%). These experiments indicate that alkaline isomerization and GLC data lead to similar results.

To find a mathematical relationship between the tissue lipid fatty acids involved that might show a sharper break at the point of dietary linoleate requirement, the percentage of 20:3ω9 × the percentage of 20:4ω6 in the tissue lipid was calculated. This relationship, when plotted against the dietary linoleate, resulted in the curves also shown in figure 1. The maxima for both the GLC and isomerization data were near 1.75% of calories as dietary linoleate. This indicated that as dietary linoleate was increased, the tissue 20:4ω6 increased faster than the 20:3ω9 decreased, until the dietary requirement was reached. At that point, the tissue 20:3ω9 decreased at a faster rate than the 20:4ω6 increased, with a continued increase in dietary linoleate. This relationship indicated that the maximum fell near the same point taken as the

dietary linoleate requirement from the triene-to-tetraene ratio. Although these curves were more erratic, the triene × tetraene constant might have some value in locating the point of dietary linoleate requirement. Similar curves calculated from tissue lipid fatty acid data from other species showed a like relationship, but tended to have a maximum at a somewhat lower value than the heretofore assigned dietary linoleate requirement for that species.

The data also demonstrated that increased dietary linoleate inhibited the conversion of oleate to 20:3ω9 in the liver lipid of the chicks. This substantiates for a third species the observation that increased dietary linoleate inhibits the metabolic conversion of oleate to eicosatrienoate, an inhibition previously shown for guinea pigs (21) and rats (22, 23).

Minimal nutritional requirements were calculated by computer methods (24) from the rate constant in the exponential equations of the form:

$$Y = a + be^{-rx}$$

where Y is the % of tissue fatty acid measured, a, b and r are constants derived, and x is the dietary linoleate as % of calories. The minimum nutritional requirement was estimated from the rate constant, r, by the equation:

$$MNR = 1.2/r$$

The MNR values for the deposition of 18:2ω6 in liver measured from GLC data was found to be 1.02% of calories. The requirement to suppress 20:3ω9 in liver was found to be 1.02% of calories. The requirement for synthesis and deposition of 20:4ω6 in liver was found to be 2.7% of calories. The MNR values derived from isomerization data were slightly higher, but we regard GLC data to be more precise.

Because we believe that the decrease of 20:3ω9 is a more important index of EFA status than is the excessive accumulation of 20:4ω6 in response to high dose levels of 18:2ω6, the MNR value of 2.7 for the latter function may be too high. Therefore, we feel that 2% of linoleate calories is sufficient to meet the more important and essential biochemical functions of the chick.

These data permit prediction of the dietary linoleate intake from the measurement of tissue fatty acid composition. Following the least squares method of Caster et al. (25), equations were computed for the estimate of linoleate intake from analysis of liver fatty acids. From experiment 1 in which analyses were by alkaline isomerization the relationship is:

$\log_{10}$ dietary linoleate $= -1.560 + 0.0756 (A - B + C + 10)$, where A is dienoic acid, B is trienoic acid and C is tetraenoic acid in % of total fatty acids. The correlation coefficient is $R = 0.888$.

From experiment 2 in which analyses were by GLC, the relationship is:

$\log_{10}$ dietary linoleate $= -1.232 + 0.0396 (A - B + C + 10)$, where A is 18:2ω6, B is 20:3ω9 and C is 20:4ω6 in % of total fatty acids. The correlation coefficient is $R = 0.907$.

The higher value of R for the GLC data indicates that this method is the better analytical procedure. The estimation equation provides a method of calculating the dietary linoleate level fed to chicks when the previous diet is not known or is unavailable for analysis. The accuracy of the estimate is within perhaps 25%.

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Effect of Chelating Agents and High Levels of Calcium and Phosphorus on Bone Calcification in Chicks Fed Isolated Soy Protein [1]

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ABSTRACT In an attempt to gain a better understanding of the rachitogenic activity of isolated soybean protein, experiments were conducted to determine relative tibia uptake of intramuscular doses of ^{45}Ca and ^{32}P in chicks fed isolated soybean protein or diets previously shown to improve bone calcification and to determine the effect of various chelating agents and high levels of calcium or phosphorus, or both, on bone calcification in chicks fed the rachitogenic diet (40% isolated soybean protein). All diets contained levels of phosphorus, calcium and vitamin D_3 higher than those recommended by the National Research Council for chicks. Tibia uptake of intramuscularly injected ^{45}Ca and ^{32}P generally was higher in chicks fed diets that improved bone calcification. None of the chelating agents (varying in stability constants for calcium from 1.6 to 10.6) improved bone calcification or growth rate. Two agents with calcium stability constants of 4.8 and 6.4 significantly depressed growth and percentage tibia ash. Adding calcium or phosphorus alone failed to improve bone calcification but a combination resulted in a significant improvement. However, tibia ash of chicks fed levels of 2.68% calcium and 1.45% phosphorus was still significantly less than that of chicks fed a diet with casein-gelatin substituted for isolated soybean protein.

O'Dell and Savage [4] first demonstrated that isolated soybean protein interfered with trace mineral utilization when they found that the zinc requirement of chicks fed a semipurified diet containing this protein was considerably greater than for chicks fed a diet containing casein and gelatin. Interference with the utilization of manganese and copper, as well as zinc, was observed in chicks fed soy protein by Davis et al. (1). Gastrointestinal absorption of ^{59}Fe in monkeys was significantly lower in the presence of isolated soybean protein than in the presence of casein (2). That isolated soybean protein also interfered with molybdenum absorption was indicated by Reid et al. (3). Zinc requirement of turkey poults fed a diet containing isolated soybean protein was reduced by adding ethylenediaminetetraacetic acid (EDTA) or by autoclaving the protein (4). EDTA improved the utilization of manganese, copper and zinc in chicks receiving a similar diet (1). Chelating agents with a stability constant for zinc between 13 and 17 were found to be the most satisfactory for growth promotion in turkey poults fed a diet containing soy protein (5).

That isolated soybean protein may interfere with macro-elements was indicated by the work of Carlson et al. (6, 7) who reported that turkey poults became rachitic when fed a semipurified diet containing a high level of this protein. Bone calcification could be improved by autoclaving the isolated soybean protein, greatly increasing the vitamin D level in feed, or by substituting soybean meal for part of the isolated soybean protein. An unidentified antirachitic factor in soybean meal was postulated (7). Chicks fed a diet containing 40% isolated soybean protein also

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[4] O'Dell, B. L., and J. E. Savage 1957 Symptoms of zinc deficiency in the chick. Federation Proc., *16*: 394 (abstract).

had subnormal bone ash, which could be partially corrected by autoclaving the protein and greatly increasing the vitamin D level, or corrected completely by reducing the level of isolated soybean protein (8). Absorption of ^{45}Ca by intestinal loops of chicks previously fed isolated soybean protein was not less than that of chicks previously fed casein-gelatin diets, suggesting that there was no interference with absorption or metabolism of vitamin D.

In an attempt to gain a better understanding of the rachitogenic effect of isolated soybean protein, the experiments reported here were conducted to study the tibia uptake of an intramuscular dose of ^{45}Ca and ^{32}P in chicks fed isolated soybean protein, and to determine the effect of various chelating agents and of increasing the level of calcium and phosphorus on growth and bone ash in chicks fed this protein.

EXPERIMENTAL

Composition of the isolated soy protein basal diet and the environmental conditions for the chicks were similar to those reported previously (8). Casein and gelatin or soybean meal were substituted for isolated soy protein and carbohydrate so that the diets were isonitrogenous. All other supplements were added to the basal diet. One-day-old Single Comb White Leghorn male chicks were used. In experiments 1 and 2, the chicks were fed the basal diet for 3 weeks and then the experimental diets for one week before being dosed with ^{45}Ca and ^{32}P. Fifteen chicks fed each diet in experiment 1, and 12 chicks in experiment 2 were dosed intramuscularly with 20 μCi ^{45}Ca (0.8 mg Ca) and 30 μCi ^{32}P (0.0003 mg P) in 0.5 ml of distilled water per chick and killed 16 hours later for removal of the left tibia. Lights were turned off for 10 hours each night in experiment 1, but in experiment 2 all birds were given continuous light during the experimental feeding period. Procedures for determining radioactivity in the tibia and bone ash were described previously (8).

In experiment 3, chicks were fed the basal diet for 14 days and then various levels of EDTA for 14 days. In experiment 4, the chicks were fed the basal diet for 7 days and then various chelating agents for 21 days. The chelating agents included EDTA, hydroxyethylethylenediaminetriacetic acid (HEDTA), nitrilotriacetic acid (NTA), (2-hydroxyethylimino) diacetic acid (HEIDA), 8-hydroxy 5-quinoline-sulfonic acid (HOS) and glutamic acid (GA). The chelating agents covered a wide range (1.6 to 10.59) of stability constants for calcium and were fed at a level of 0.684 mmole/kg of diet. In experiments 5 and 6, chicks were fed the basal diet for 21 days and the experimental diets for 9 days. Precipitated calcium carbonate served as the source of supplementary calcium in experiments 7 and 8. The combined phosphorus and calcium supplements in experiment 7 were supplied by defluorinated rock phosphate. All of the supplementary phosphorus in experiment 8 was supplied by phosphoric acid. In experiments 7 and 8, the chicks were fed the basal diet for 14 days followed by the experimental diets for 14 days. In experiments 3–8, three groups of 8 chicks were fed each experimental diet.

RESULTS

Uptake of an intramuscular dose of ^{32}P in the tibia was significantly greater for birds fed the basal diet supplemented with 20% soybean meal (table 1), but uptake of ^{45}Ca was not significantly different. In experiment 2, tibia uptake of ^{45}Ca was significantly higher for birds fed a casein-gelatin diet than for those fed the isolated soybean protein diet (table 2). Although a higher percentage of the dose of ^{32}P was taken up by the tibia of chicks fed the casein-gelatin and autoclaved soy protein diets, the values were not significantly different ($P > 0.05$) from those for the chicks fed the basal diet. In both experiments, no significant difference among treatments was evident when uptake of dose of either ^{45}Ca or ^{32}P was related to content of tibia ash. Therefore, the observed differences in tibia uptake probably reflect only differences in mineral mass with which isotope exchange occurred.

Levels of EDTA from 0.02 to 2% did not influence the percentage tibia ash, but the highest level significantly depressed growth (table 3). Chelating agents varying in stability constants for calcium from

TABLE 1

Effect of soybean meal on tibia uptake of an intramuscular dose of ^{45}Ca and ^{32}P (exp. 1)

	Basal	Basal + 20% soybean meal
Average body weight, g	318	342 [1]
Average tibia ash, g	0.399	0.449 [1]
Tibia uptake of ^{45}Ca		
% of dose	4.87	4.98
% of dose/g tibia ash	12.56	11.26
% of dose/100 mg tibia calcium	3.48	3.12
Tibia uptake of ^{32}P		
% of dose	4.05	4.54 [1]
% of dose/g tibia ash	10.58	10.30

[1] Significantly different ($P < 0.05$) from chicks fed basal diet.

TABLE 2

Effect of diet on tibia uptake of an intramuscular dose of ^{45}Ca and ^{32}P (exp. 2)

	Diet		
	Soy protein	Autoclaved soy protein	Casein-gelatin
Average body weight, g	342 [a 1]	353 [a]	322 [a]
Average tibia ash, g	0.419 [A]	0.455 [B]	0.472 [B]
Tibia uptake of ^{45}Ca			
% of dose	4.88 [A]	5.24 [AB]	5.66 [B]
% of dose/g tibia ash	11.64 [a]	11.52 [a]	11.99 [a]
Tibia uptake of ^{32}P			
% of dose	5.44 [a]	5.57 [a]	5.81 [a]
% of dose/g tibia ash	12.98 [a]	12.37 [a]	12.31 [a]

[1] Duncan's (15) multiple range test; upper case letter, $P < 0.01$, and lower case letter, $P < 0.05$. Values followed by the same letter are not significantly different.

TABLE 3

Effect of dietary level of EDTA on growth and tibia ash of chicks (exp. 3)

EDTA	Avg gain (14 days)	Tibia ash
%	g	%
0	126 [b 1]	36.8 [a 1]
0.02	127 [b]	37.7 [a]
0.2	140 [b]	37.2 [a]
2.0	77 [a]	37.7 [a]

[1] Duncan's (15) multiple range test ($P < 0.05$). Values followed by the same letters are not significantly different.

1.6 to 10.59 did not increase tibia ash of chicks over the unsupplemented basal ration (table 4). NTA and HEIDA significantly depressed bone ash and growth rate.

Substituting 10% soybean meal in the basal diet significantly improved bone ash, but ash of soybean meal equivalent to 10% meal did not (table 5). Extra phosphorus in the form of potassium acid

TABLE 4

Effect of various dietary chelating agents on growth and tibia ash of chicks (exp. 4)

Supplement	Ca stability constant	Avg gain (21 days)	Tibia ash
%		g	%
None	—	201 [cd 1]	38.6 [c 1]
0.2 EDTA [2]	10.6	192 [c]	37.9 [c]
0.19 HEDTA [3]	8.0	189 [bc]	39.0 [c]
0.13 NTA [4]	6.4	152 [a]	36.7 [b]
0.121 HEIDA [5]	4.8	168 [ab]	35.3 [a]
0.154 HQS [6]	3.5	183 [bc]	37.9 [c]
0.1 GA [7]	1.6	185 [bc]	38.4 [c]
Casein-gelatin	—	159 [a]	47.0 [d]

[1] Duncan's (15) multiple range test ($P < 0.01$). Values followed by the same letter are not significantly different.
[2] EDTA indicates ethylenediaminetetraacetic acid.
[3] HEDTA indicates hydroxyethylethylenediaminetriacetic acid.
[4] NTA indicates nitrilotriacetic acid.
[5] HEIDA indicates (2-hydroxyethylimino) diacetic acid.
[6] HQS indicates 8-hydroxy 5-quinoline-sulfonic acid.
[7] GA indicates glutamic acid.

phosphate did not improve growth or bone ash significantly. In experiment 7, increasing the level of calcium in the diet had a variable effect on growth rate but significantly depressed percentage tibia ash (table 6). However, adding more calcium and phosphorus in the form of defluorinated rock phosphate significantly improved growth and bone calcification. In experiment 8, levels of calcium or phosphorus fed individually failed to increase either growth or bone ash (table 7). The highest level of phosphorus significantly depressed growth rate and the highest level of calcium significantly depressed tibia ash. Adding the 2 minerals together significantly increased bone ash over the basal diet even though growth rate was not greatly affected.

TABLE 5

Effect of soybean meal ash and additional phosphate on growth and tibia ash of chicks (exps. 5 and 6)

Supplement	Avg gain (9 days)	Tibia ash
	g	%
None	92 [a 1]	39.1 [a 1]
10% soybean meal	106 [a]	41.1 [b]
Ash ≅ 10% soybean meal	87 [a]	39.3 [a]
None	98 [a]	38.1 [a]
0.5% KH_2PO_4	101 [a]	39.3 [a]
1.0% KH_2PO_4	93 [a]	37.6 [a]

[1] Duncan's (15) multiple range test ($P < 0.05$). Values followed by the same letter are not significantly different.

TABLE 6

Effect of additional calcium and phosphorus and of levels of calcium on growth and tibia ash of chicks (exp. 7)

Supplement		Avg gain (14 days)	Tibia ash
Ca	P		
%	%	*g*	%
0	0	153 [a 1]	40.3 [c 1]
0.3	0	172 [b]	36.9 [b]
0.6	0	169 [ab]	37.3 [b]
1.2	0	153 [a]	33.4 [a]
1.2	0.6	192 [c]	43.5 [d]

[1] Duncan's (15) multiple range test ($P < 0.01$). Values followed by the same letter are not significantly different.

DISCUSSION

In a study reported elsewhere (9), it was observed that isolated soybean protein markedly interfered with absorption of both calcium and phosphorus in chicks, as compared with those fed a diet containing casein and gelatin. The results reported here show that increasing the level of either calcium or phosphorus individually failed to increase bone calcification. In fact, increasing levels of calcium significantly reduced tibia ash. However, increasing both calcium and phosphorus at the same time significantly improved bone calcification. Even with a level of 2.68% calcium and 1.45% phosphorus, however, tibia ash was not as high as that obtained by substituting casein-gelatin for the isolated soybean protein (table 7).

The calcification problem studied here is analogous to the problem of zinc availability in diets containing isolated soybean protein. In both cases isolated soybean protein markedly interferes with the ab-

TABLE 7

Effect of levels of calcium and phosphorus alone and in combination on growth and tibia ash of chicks (exp. 8)

Supplement		Diet analysis		Avg gain (14 days)	Tibia ash
Ca	P	Ca	P		
%	%	%	%	*g*	%
0	0	1.44	0.94	143 [bc 1]	37.8 [abc 1]
0.3	0	1.72	1.01	153 [c]	38.7 [bc]
0.6	0	2.06	0.94	139 [bc]	35.7 [ab]
1.2	0	2.59	0.89	133 [b]	35.0 [a]
0	0.15	1.55	1.10	133 [b]	39.2 [c]
0	0.30	1.51	1.27	131 [b]	38.1 [bc]
0	0.60	1.54	1.47	113 [a]	37.1 [abc]
0.3	0.15	1.85	1.07	147 [bc]	42.0 [d]
0.6	0.30	2.17	1.24	148 [bc]	42.4 [c]
1.2	0.60	2.68	1.45	138 [bc]	43.1 [d]
Casein-gelatin		1.72	1.00	142 [bc]	45.8 [e]

[1] Duncan's (15) multiple range test ($P < 0.05$). Values followed by the same letter are not significantly different.

sorption of the minerals in comparison with diets containing protein from animal sources. The zinc availability problem can be corrected by autoclaving the isolated soybean protein (4), or by greatly increasing the zinc level in the diet. Similarly, autoclaving the isolated soybean protein and increasing the levels of calcium and phosphorus partially overcome the bone calcification problem. Evidence has been presented that the factor in isolated protein interfering with zinc utilization is phytic acid (10). It is possible that phytic acid is also interfering with calcium and phosphorus absorption. Evidence that phytic acid interferes with calcium absorption in dogs and humans was published several years age (11, 12). The rachitogenic effect of phytic acid in dogs, however, could be counteracted by adding calcium alone. This is in contrast with the results reported here with chicks where supplements of both calcium and phosphorus were necessary to improve calcification. Evidence that phytic acid per se was not interfering with calcification was presented by Likuski and Forbes (13). They observed that adding phytic acid to either a casein or amino acid diet for chicks significantly decreased availability of zinc but did not significantly affect femur ash. It has long been known that the phosphorus in phytate is less available than that in inorganic form for animals. If the phytate in isolated soybean protein is responsible for the reduced absorption of calcium and phosphorus, this suggests that phytic acid must also interfere with availability of inorganic phosphorus.

Although EDTA improved availability of zinc from isolated soybean protein diets for poults and chicks, it apparently has no influence on calcium availability. Even though EDTA has a strong affinity for calcium and it is apparently absorbed (14), a level as high as 2% in the diet did not change tibia ash from that of birds fed the unsupplemented basal diet. The chelating agents, NTA and HEIDA, however, with stability constants in the 4 to 6 range for calcium, significantly depressed bone ash. The mechanism for this depression is unknown but it could be that these agents are not absorbed and, therefore, reduce absorption of calcium from the alimentary tract. The fact that none of the chelating agents used in this study improved bone calcification over that obtained with the basal diet may be related to the need for increased absorption of both calcium and phosphorus to bring about an improvement in calcification.

It was thought that the intramuscular injection of radioactive calcium and phosphorus might give information as to which of the elements was most limiting in the diet. It was hypothesized that there may be a greater uptake in the tibia of one or both of these isotopes in birds fed the basal ration where absorption of the minerals was interfered with, than in birds fed a diet containing casein or supplemented with soybean meal. Generally, however, a greater uptake was observed with the supplemented diets than with the basal diet. In view of the marked excretion of endogenous calcium and phosphorus into the digestive tract, (9) it is likely that considerable radioactive calcium and phosphorus were also excreted into the tract. Once in the tract, these radioactive minerals would be subject to the same dietary factors affecting absorption as dietary calcium and phosphorus.

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Fatty Acid Changes in Liver Produced by Protein Deficiency and by Methionine or Cystine Fed to Rats in a Protein-free Ration

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ABSTRACT Liver fatty acid changes were studied during the development of an uncomplicated protein deficiency and in protein deficiency complicated by the addition of 0.30% methionine to the protein-free diet. (Only slightly fatty livers are produced in uncomplicated protein deficiency, whereas very fatty livers are produced by including methionine or cystine in a protein-free diet.) In general the fatty acid changes in adult male rats fed either diet followed similar patterns, although the extent of the changes was different. The increase in liver fatty acids could be accounted for almost entirely by elevations in palmitate, oleate, and linoleate. When dietary fat (corn oil) was omitted, however, the contribution by linoleate was depressed almost completely, being substituted for by palmitoleate and oleate. Significant loss of all fatty acids from the phospholipid fraction occurred. The presence of methionine in the protein-free diet in general protected against the loss of fatty acids from this fraction. Palmitoleate and oleate substituted for linoleate in the phospholipid fraction when dietary corn oil was omitted. The fat in the fatty livers produced by methionine or cystine was accounted for almost entirely by marked increases in neutral lipid palmitate, oleate, and linoleate. Cystine, methionine, and valine fed in the protein-free diet increased arachidonate concentration in the neutral lipid fraction, whereas leucine depressed it almost completely. When protein repletion was begun, all fatty acids except linoleate and arachidonate returned to extremely high values in the livers of the protein-free rats and to a much less extent in livers of the rats previously fed methionine in the protein-free diet. These studies suggest the following general conclusions: An as yet unknown mechanism allows the production of fatty livers when methionine is present in a protein-free ration. If linoleate is present in the diet, it accounts for a large part of the fat; but palmitoleate and oleate can substitute for linoleate. An imbalance between fat-synthesizing systems and fat-secreting systems in the liver probably accounts for a very large, transient increase in fatty acid concentrations when protein is reintroduced into the diet of protein-deficient animals. The enzyme systems involved in producing arachidonate from linoleate appear to be sharply impaired by protein deficiency. Methionine fed in a protein-deficient diet, however, almost completely prevents this effect.

In a previous publication of this series (1) it was reported that protein deprivation produced slightly fatty livers in adult male rats. When 0.30% methionine or equivalent cystine was included in the protein-free ration, however, very fatty livers were produced. A peak in neutral lipid concentration was reached after 8 weeks with a tendency to return to normal thereafter. The increased fat could be accounted for entirely as neutral glycerides and cholesterol. While phospholipid concentration per liver cell decreased significantly in both groups, methionine prevented further loss after 3 weeks of protein deprivation (2). Cystine was also active in this respect.

The present study is part of a continuing investigation of changes in intracellular components closely related to the maintenance of energy metabolism during prolonged protein deprivation. Phospholipids have been found to be necessary for the activity of several segments of the electron-transport system in mitochondria (3–6). Neutral lipids and phospholipids of any one species of animals fed a constant diet are known to contain, within limits, definite ratios of saturated and non-saturated fatty acids. Whether these ratios change in an uncomplicated protein deficiency or in fatty livers produced by addition of me-

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thionine or cystine to a protein-free ration is unknown, however.

In the present paper are reported the changes in liver fatty acids associated with the aforementioned dietary conditions as well as during the return to normal during protein repletion. We also wish to report a comparison of phospholipid and neutral lipid fatty acids at the time of the peak effect of the deficient diets, which was 8 weeks after the introduction of the deficiencies.

EXPERIMENTAL METHODS

The study was divided into 2 phases. First, the changes in liver fatty acids associated with prolonged protein depletion were followed during progressive protein depletion and the subsequent period of repletion. The effect of feeding 0.30% methionine in the protein-free ration was also studied. In the second phase the effects of adding individually those other amino acids which have been shown in earlier papers (1, 2) to influence liver lipid concentrations were studied. Also, in the second phase, the influence of omitting all dietary fat on the fatty acid composition of neutral lipids and phospholipids in rats fed the complete control diet, the protein-free diet, and the protein-free diet with added methionine was studied. Since the composition of the fatty acids in the corn oil normally supplied in the diet was known, the results of this study should give information concerning to what extent extra-hepatic tissues supply essential fatty acids to the neutral lipids and phospholipids of the liver in protein deficiency or in a fatty liver produced by methionine or cystine fed in a protein-deficient ration.

The feeding and management of the animals have been presented in detail in a preceding paper (1). The livers used for analysis in the present study were the same as those used in the preceding studies for neutral lipids, cholesterol, phospholipids and plasmalogens (1, 2).

All of the rats (male adults of the Sprague-Dawley strain) were fed the complete diet for a 3-week adjustment period after which they weighed 290 g. Briefly, the complete diets consisted of: (in per cent) casein, 20; DL-methionine, 0.30; corn oil, 5; salts N plus molybdate (7), 6.5; glucose monohydrate, 63.5; choline chloride, 0.2; *i*-inositol, 0.02; and water-soluble vitamins mixed in sucrose (8), 4.5. Fat-soluble vitamins (9) were given weekly to each rat in 2 drops of corn oil.

In phase 1 four groups of rats were included: group 1, ad libitum-fed controls; group 2, pair-fed controls (fed average daily food consumption of group 4); group 3, protein-deficient group with 0.3% DL-methionine included in the ration; and group 4, protein-deficient group. In phase 2, the effects of cystine, methionine, leucine, and valine added individually to the protein-free ration upon the fatty acids of phospholipids and neutral lipids were studied. These were the amino acids shown to have the most pronounced effect upon liver lipids. The diets in this phase were fed for 8 weeks.

Liver lipids were extracted by the procedure of Folch et al. (10); and liver DNA by the extraction procedure of Schneider (11) followed by color development with diphenylamine (12). Total lipids were assayed by the method of Bragdon (13), phospholipid by the method of Fiske and Subbarow (14), and total cholesterol by the method of Pearson et al. (15). Gas-liquid chromatography of the fatty acids in the liquid extracts was accomplished by the procedure outlined by Bieri and Andrews (16). In this procedure the methyl esters of the fatty acids were first prepared by transesterification with methanol using BF_3 as catalyst. The esters were then separated by gas-liquid chromatography on a 15% ethylene-glycol succinate column in a Barber Colman, Model 15, instrument. Neutral lipids were separated from phospholipids by the following procedure: Folch extract equivalent to 200 mg of liver was evaporated to dryness under nitrogen at 60°. The residue was dissolved in 5.5 ml chloroform. Five milliliters of solution were applied to a small chromatographic column (0.75 cm I.D. × 14 cm) containing 1.2 g of silicic acid [1] previously washed 4 times with chloroform. The solvent was pulled through slowly by gentle suction, approximately 1 drop/second. The eluate was collected in a graduated centrifuge tube. When the solvent was almost through (about 3 mm from the top of the silicic

[1] Mallinkrodt Chemical Works, Philadelphia.

acid), 10 ml of chloroform were added to wash the adsorbed phospholipids free of neutral lipids. The wash eluate was collected in the same tube as the first eluate. This tube then contained the neutral lipids. The phospholipids were eluted by passing 15 ml methanol through the column and collecting the eluate. Fatty acids of the separated neutral lipid and phospholipid fractions were analyzed as above, using gas-liquid chromatography.

Calculation from total lipid, phospholipid, and total cholesterol data of each fatty acid as micrograms or as micromoles of fatty acid per unit weight of liver was carried out using equations based on the following assumptions: 1) The fatty acid content of liver lipid extracts is composed mainly of fatty acids from neutral glycerides, phospholipids, and cholesterol esters, and free fatty acids. 2) The average molecular weight of fatty acids in liver is approximately 282 (obtained from the ratios in normal rat liver). 3) Total lipid is measured by Bragdon's method (13) using stearate standard expressed in micromoles. 4) Lecithin and cephalin concentrations are equal, each making up 47.5% of the total phospholipids. 5) Sphingomyelin concentration is 5% of the total phospholipids. 6) The average molecular weight of phospholipids is 760. 7) The free cholesterol-to-cholesterol ester ratio is 4:1. 8) Free fatty acids are in negligible concentration in liver. (The influence of variations in these assumptions will be discussed later.) Based on these assumptions the following equation can be derived (see appendix):

μg of fatty acids/unit weight of liver =
255 μmoles total lipid/unit wt of liver
145 μmoles phospholipid/unit wt of liver
341 μmoles total cholesterol/unit wt of liver

Converting the percentage of any one fatty acid from gas-liquid chromatographic data to micromoles of that fatty acid per unit weight of liver is accomplished by the following equation:

μmoles of any fatty acid/unit weight of liver =

$$\frac{\%\text{ of that fatty acid (from GLC)}}{\mu\text{mol. wt of that fatty acid}}$$
[2.55 μmoles total lipid/unit wt of liver — 1.45 μmoles phospholipid/unit wt of liver — 3.41 μmoles total cholesterol/unit wt of liver]

To calculate neutral lipid or phospholipid fatty acid per milligram of liver DNA from GLC data using neutral lipid or phospholipid fractions separated on silicic acid the following equations, which are derived from the preceding assumptions and equations, are used:

μmoles of any fatty acid in neutral lipid fraction/mg DNA =

$$\frac{\%\text{ of that fatty acid (from GLC)}}{\mu\text{mol. wt of that fatty acid}}$$
[2.55 μmoles total lipid/mg DNA — 6.81 μmoles phospholipid/mg DNA — 3.41 μmoles total cholesterol/mg DNA]

μmoles of any phospholipid fatty acid/mg DNA =

$$\frac{\%\text{ of that fatty acid (from GLC)}}{\mu\text{mol. wt of that fatty acid}}$$
[5.37 μmoles phospholipid/mg DNA]

The total lipid, phospholipid, and total cholesterol values in the preceding 2 equations are obtained from the unseparated lipid extract; the GLC values, from the fractions separated on silicic acid.

These equations are very useful when total lipid, phospholipid, and total cholesterol data are available but not total fatty acid data. If total fatty acid data are available, for example, after saponification and direct titration of the fatty acids, calculations of micromoles of individual fatty acids per milligram of DNA from GLC data can be made directly. If total lipid data are obtained from evaporation of solvent from lipid extracts and weighing of the residue, the above equations are equally useful. In each of the equations is substituted micrograms of total lipid per unit weight of liver or per milligram of DNA in place of 255 (or 2.55) μmoles of total lipid.

It can be calculated that errors arising from variations in the assumptions used to obtain the above equations may give rise to the following errors, as examples: 1) If cholesterol is present entirely as ester, the error is − 0.6%. 2) If sphingomyelin is increased from 5 to 25% of the phospholipid, the error is +5%. 3) If cephalin is increased from 47.5 to 77.5% and lecithin is decreased to 17.5%, the error is −2%. 4) If free fatty acids are increased from zero to 10% of the total lipids, the error is −1.5%. It is doubtful whether variations from the assumptions larger than these

will be encountered, and thus the equations are reasonably valid over a wide range of variability among lipid components.

RESULTS

The results are presented as micromoles of fatty acid per milligram of liver DNA. Since the concentration of DNA per average liver cell nucleus has been found to be constant during protein deprivation in adult rats (17), concentration of liver fatty acids expressed per milligram of DNA should be directly proportional to concentration per average cell.

Phase 1. The results of this phase are presented in figures 1–3. The ad libitum-fed control group (group 1) and pair-fed (group 2) have been separated from the 2 protein-deficient groups (groups 3 and 4) for clarity. The results for fatty acids that can be synthesized *de novo* by the rat are presented in figures 1 and 2, while the results for linoleic and arachidonic are presented in figure 3. Myristic and linolenic acids were also measured but the results are not presented since the values were negligible compared with the other acids and also showed no changes of significance.

The rats fed the protein-free diet plus 0.3% DL-methionine (group 3) showed a five- to sixfold increase in concentration of palmitate, oleate and linoleate during the first 8 weeks of deficiency, followed by a decrease toward normal as the deficiency continued. Both palmitoleic and stearic acids increased two- to threefold in the methionine-fed group, whereas arachidonic acid decreased only slightly. In the protein-deficient group not fed methionine (group 4) an increased concentration of fatty acids occurred only in palmitic, oleic, and linoleic acids. These increases were not nearly as great as in group 3, however. There were no changes of significance in either the ad libitum-fed normal control group (group 1) or in the pair-fed control group (group 2). When protein was reintroduced into the ration after 14 weeks of deficiency, all of the fatty acid values except for linoleic acid returned to extremely high levels in group 4, whereas in the protein-deficient group formerly fed methionine the fatty acids rebounded only slightly. After these initial rapid changes both of the formerly protein-deficient groups returned to normal after 8 weeks of repletion.

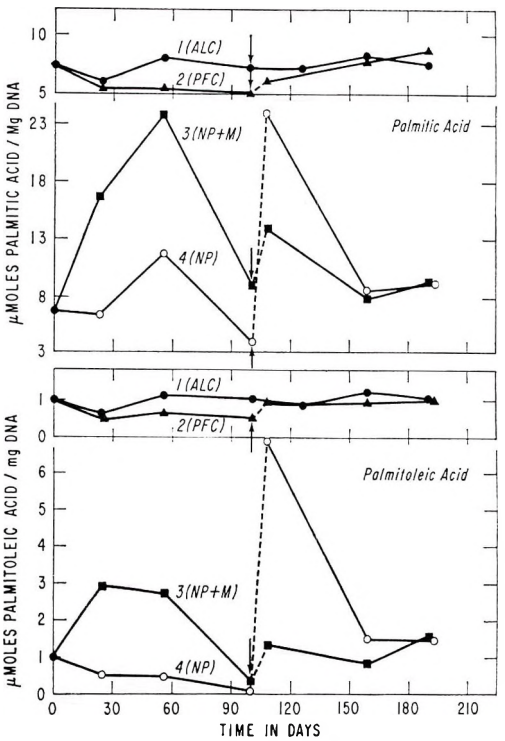

Fig. 1 The response of liver palmitic and palmitoleic acids to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. (1, ALC), ●, indicates ad libitum-fed controls; (2, PFC), ▲, pair-fed controls; (3, NP + M), ■, protein-deficient rats supplemented with 0.30% DL-methionine; (4, NP), ○ (small open circles), protein-deficient rats. Repletion was begun at the arrows. Statistical analysis using Student's *t* test indicates that the following points are significantly different from each other, with $P < 0.01$: *Palmitic acid:* 56 days, 1 vs. 3, 2 vs. 3, 2 vs. 4, and 3 vs. 4; zero to 15 days post-repletion, 1 vs. 4 and 2 vs. 4. *Palmitoleic acid:* 24 days, 1 vs. 3, 2 vs. 3, and 3 vs. 4; 56 days, 1 vs. 3, 2 vs. 3, and 3 vs. 4; 102 days, 1 vs. 4, and 2 vs. 4; zero to 15 days post-repletion, 1 vs. 4 and 2 vs. 4.

Phase 2. In table 1 are presented the results of the gas-liquid chromatography of fatty acids in the neutral lipid fraction and phospholipid fractions after separation on silicic acid. It should be reemphasized that these results were obtained after 56 days of protein depletion which was the time when the maximal effects on neutral lipids occurred.

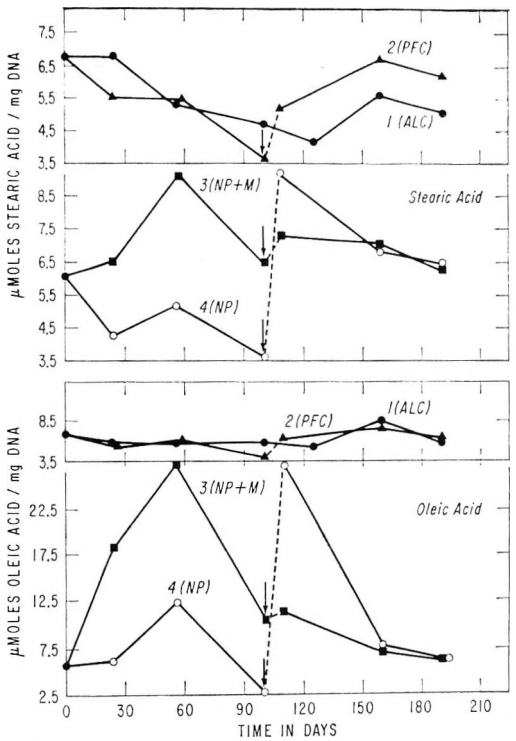

Fig. 2 The response of liver stearic and oleic acids to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. (1, ALC), ●, indicates ad libitum-fed controls; (2, PFC), ▲, pair-fed controls; (3, NP + M), ■, protein-deficient rats supplemented with 0.30% DL-methionine; (4, NP), ○ (small open circles), protein-deficient rats. Repletion was begun at the arrows. Statistical analysis using Student's *t* test indicates that the following points are significantly different from each other, with $P < 0.01$: *Stearic acid:* 24 days, 1 vs. 4; 56 days, 1 vs. 3, 2 vs. 3, and 3 vs. 4; zero to 15 days post-repletion, 1 vs. 3, 1 vs. 4, and 2 vs. 4. *Oleic acid:* 24 days, 1 vs. 3; 56 days, 1 vs. 3, 1 vs. 4, 2 vs. 4, and 3 vs. 4; zero to 15 days post-repletion, 1 vs. 4 and 2 vs. 4.

In uncomplicated protein deficiency (the control protein-deficient group in the table), significant loss of all fatty acids from the phospholipid fraction occurred. Protection against this loss was given by cystine and in most cases (except for arachidonate and stearate) by methionine. These results fit well with previous results (2) in which it was found that total phospholipids are lost in uncomplicated protein deficiency but are somewhat protected against loss by methionine and cystine. None of the other amino acids tested showed any effect in this respect. Methionine and cystine increased phospholipid linoleate to levels significantly above normal. This was the only phospholipid fatty acid which responded in this manner. When corn oil was omitted from the diet, the same general effect on phospholipid fatty acids was observed: In uncomplicated protein deficiency each fatty acid was lost from the cells; and in most cases (again except for arachidonate and stearate) methionine protected against this loss. When corn oil was omitted,

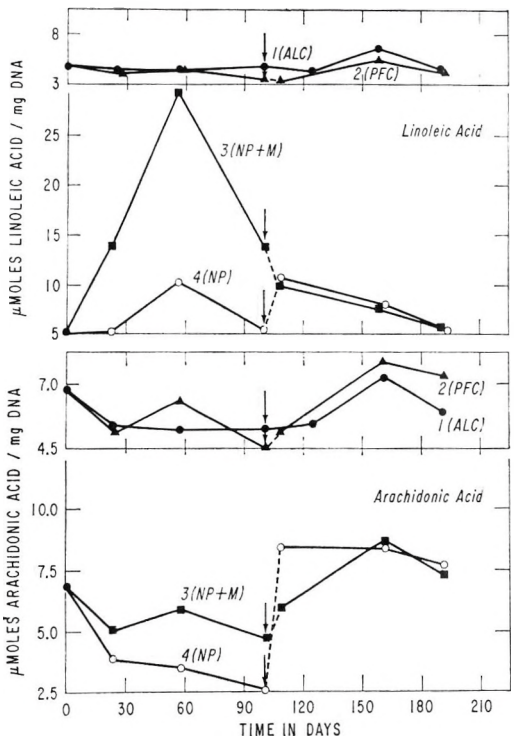

Fig. 3 The response of liver linoleic and arachidonic acids to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. (1, ALC), ●, indicates ad libitum-fed controls; (2, PFC), ▲, pair-fed controls; (3, NP + M), ■, protein-deficient rats supplemented with 0.30% DL-methionine; (4, NP), ○ (small, open circles), protein-deficient rats. Repletion was begun at the arrows. Statistical analysis using Student's *t* test indicates that the following points are significantly different from each other, with $P < 0.01$: *Linoleic acid:* 24 days, 1 vs. 4 and 2 vs. 4; 56 days, 1 vs. 3, 1 vs. 4, 2 vs. 3, 2 vs. 4, and 3 vs. 4; 102 days, 1 vs. 2; zero to 15 days post-repletion, 1 vs. 4 and 2 vs. 4. *Arachidonic acid:* 24 days, 1 vs. 4; 56 days, 1 vs. 4, 2 vs. 4, and 3 vs. 4; 102 days, 1 vs. 2, 1 vs. 4, and 2 vs. 4.

TABLE 1

Response of liver neutral lipid and phospholipid fatty acids to 56 days of feeding rats a protein-free ration with and without various amino acids

	With corn oil in diet					Without corn oil in diet		
	Ad libitum-fed normal controls	Protein-deficient rats				Ad libitum-fed normal controls	Protein-deficient rats	
		Control group	Fed 0.24% L-cystine	Fed 0.30% L-methionine	Fed 0.26% L-leucine	Fed 0.24% valine		Control group	Fed 0.30% L-methionine
	Neutral lipid fatty acids, μmoles/mg DNA								
Palmitate	4.61 ± 0.5 [1]	7.43 ± 0.8	41.3 ± 8.3	18.8 ± 1.3	3.66 ± 0.3	12.2 ± 2.4	1.79 ± 0.21	7.17 ± 1.1	44.2 ± 10
Palmitoleate	0.87 ± 0.07	0.70 ± 0.2	3.75 ± 1.1	2.07 ± 0.7	0.47 ± 0.06	0.57 ± 0.2	0.60 ± 0.08	1.94 ± 0.6	11.6 ± 1.8
Stearate	0.71 ± 0.1	1.28 ± 0.2	6.60 ± 1.0	3.28 ± 1.0	0.76 ± 0.09	2.71 ± 0.5	0.36 ± 0.007	1.17 ± 0.2	6.56 ± 0.4
Oleate	5.44 ± 0.7	10.1 ± 1.2	52.0 ± 7.4	25.6 ± 1.9	4.19 ± 0.4	17.8 ± 3.7	3.22 ± 0.1	12.7 ± 2.1	69.3 ± 14
Linoleate	2.63 ± 0.4	7.44 ± 1.0	37.5 ± 7.0	19.9 ± 3.5	3.53 ± 0.4	13.5 ± 2.8	0.43 ± 0.03	0.81 ± 0.007	3.10 ± 1.5
Arachidonate	1.16 ± 0.1	0.96 ± 0.1	3.02 ± 0.8	1.83 ± 0.2	0.22 ± 0.2	1.82 ± 0.1	0.78 ± 0.2	0.58 ± 0.5	0.56 ± 0.3
	Phospholipid fatty acids, μmoles/mg DNA								
Palmitate	5.70 ± 0.9	3.42 ± 0.2	5.22 ± 0.7	4.40 ± 0.2	3.67 ± 0.5	3.47 ± 0.2	6.00 ± 0.7	3.51 ± 0.5	4.98 ± 0.2
Palmitoleate	0.39 ± 0.03	0.10 ± 0.03	0.10 ± 0.04	0.2 ± 0.02	0.1 ± 0.04	0.08 ± 0.03	1.44 ± 0.13	0.48 ± 0.02	0.56 ± 0.08
Stearate	6.00 ± 0.2	3.62 ± 0.2	4.85 ± 0.6	3.85 ± 0.1	4.03 ± 0.3	3.93 ± 0.5	5.85 ± 0.4	3.39 ± 0.2	3.62 ± 0.1
Oleate	2.55 ± 0.2	1.26 ± 0.2	2.07 ± 0.6	2.14 ± 0.5	1.12 ± 0.04	1.26 ± 0.3	5.34 ± 0.4	2.45 ± 0.3	3.43 ± 1.46
Linoleate	2.88 ± 0.2	2.31 ± 0.03	4.55 ± 1.2	3.89 ± 0.8	2.54 ± 0.2	2.29 ± 0.1	1.46 ± 0.1	1.00 ± 0.08	1.40 ± 0.09
Arachidonate	7.73 ± 0.6	3.95 ± 0.16	4.62 ± 0.4	3.75 ± 0.1	3.89 ± 0.05	3.31 ± 0.3	6.30 ± 0.6	3.22 ± 0.1	3.71 ± 0.3

[1] SE of mean.

palmitoleate and oleate were considerably higher in the phospholipid fraction in all 3 groups when compared with their respective groups receiving corn oil; and linoleate was considerably lower in these groups than in the corresponding groups receiving corn oil. With all other fatty acids, however, the same results were obtained in corresponding groups in the presence and absence of dietary corn oil.

The results with the neutral lipid fractions indicate that the fat in the fatty livers produced by methionine or cystine can be accounted for almost entirely by marked increases in cellular palmitate, oleate, and linoleate. Although neutral lipid palmitoleate, stearate and arachidonate also increased several-fold, their total contribution to the total fatty acids was almost negligible compared with the other 3 fatty acids. In the control group (ad libitum-fed normal controls) (ALC) the amount of arachidonate present in the neutral lipid fraction made up about 10% of the total arachidonate (neutral lipid plus phospholipid). When cystine was added to the ration, however, arachidonate increased to 40% of the total arachidonate. Increases in neutral lipid arachidonate also occurred when either methionine or valine were present in the protein-free ration. When leucine was present, however, scarcely any arachidonate was found in the neutral lipid fraction. In fact, leucine caused a significant loss when compared with the control protein-deficient group in all neutral lipid fatty acids, which accounts for the lowered neutral glycerides observed when leucine was fed in a protein-free ration (1). Valine produced significant increases in neutral lipid palmitate, stearate, oleate, linoleate, and arachidonate, which is enough to account for the mildly fatty liver produced by valine fed in a protein-free ration reported earlier (1).

In almost every case, with the neutral lipid fatty acids in the groups receiving corn oil the effect of methionine was significantly less than the effect of cystine. However, this was due to a chance circumstance in which the lipid extracts chosen at random for fatty acid analysis from the group receiving cystine contained considerably higher total lipids than from the group receiving methionine. Thus, any neutral lipid fatty acid value per milligram of DNA would be higher from a lipid extract containing higher total lipid. In the separation of fractions on silicic acid, in all cases only 4 of a total of 8 extracts were used. Unfortunately the 4 chosen at random for the group receiving cystine contained the highest total lipid values for that group; and for the group receiving methionine, the lowest total lipid values for that group. When the average of all 8 values for total lipid along with the average of the 4 values from the GLC analysis was used in the calculation of fatty acid per milligram of DNA, the difference between the effects of cystine and methionine disappeared, the values for both groups falling at almost exactly the midpoint between the averages given in the figures. In no other groups was this problem encountered.

When corn oil was omitted from the diet, a very marked decrease in neutral lipid linoleate and arachidonate occurred in the group receiving the protein-free ration plus methionine when compared with the same group receiving corn oil. There was a marked increase in neutral lipid palmitoleate and oleate in the same group.

DISCUSSION

It is apparent from the results that the slightly fatty livers produced by uncomplicated protein deficiency and the very fatty livers produced by including methionine in a protein-free ration can be accounted for almost entirely by increases of palmitate, oleate and linoleate. In the absence of corn oil, however, although the same degree of fatty livers was obtained, the contribution by linoleate was depressed almost completely, being substituted for by palmitoleate and oleate. Thus it can be generalized that fatty livers of this type were produced in the absence or presence of dietary fat, and although the extent of fat accumulating was the same, the composition of the fat was different.

The sharp rebound of fatty acids after reintroduction of protein in the group formerly fed the protein-free ration with-

out amino acids might be explained by a difference in the rates of fatty acid or fat synthesized in the liver and fat secreted from the liver. If the fatty acid or fat-synthesizing systems either are normal at the time of protein reintroduction while there has been a loss in fat-secreting mechanism or if the fat-synthesizing systems return at a faster rate than the fat-secreting system, such a rapid increase of fat in the liver would be expected. It is of interest that the fatty acids which increased to higher than normal levels during this rebound were those which can be synthesized *de novo* by the rat. Linoleate and arachidonate simply increased to normal levels and remained there. This implies that the fatty acid-synthesizing systems are closely related to the sharp rebound of fatty acids after protein repletion. Also the fact that the fatty acid rebound was much higher in the uncomplicated protein deficiency than in the methionine-fed group upon reintroduction of protein indicates that methionine prevented to a great extent changes in the mechanisms involved in production of the rebound.

Evidence that the fatty acids in the fatty liver probably arise to a much greater extent from synthesis rather than by transport from extra-hepatic organs is that in the absence of dietary corn oil, and thus of dietary linoleate and arachidonate, all of the fatty acids except linoleate and arachidonate increased 20- to 25-fold in the neutral lipid fraction. However, linoleate increased about 7-fold in the neutral lipid fraction in the group receiving methionine, indicating that some transport from extra-hepatic organs may have occurred.

Because of the progressive decrease in arachidonate in the protein-deficient group even though linoleate increased 100% after 8 weeks of the deficiency, it appears that the enzyme systems involved in the production of arachidonate from linoleate are impaired by protein deficiency. The presence of methionine in the protein-free ration prevented this effect to some extent, since the decrease in arachidonate was less pronounced in that group. Thus it appears again that, although methionine in a protein-free ration causes a very fatty liver, it also acts to maintain certain essential liver components such as succinic oxidase, succinic dehydrogenase (18), ubiquione (19), phospholipids (2) and thus, by inference from the present studies, the enzyme systems involved in producing arachidonate from linoleate.

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APPENDIX

Using the assumptions presented in the text, keeping in mind that the final equation is to express only micrograms of fatty acids with all other lipid moieties subtracted out, and recognizing that micromoles of total lipid (expressed as stearate) $\times$ 285 = micrograms of total lipid, then the following general equation can be set up:

μg of fatty acids =

I [μmoles total lipid $\times$ 285 $-$ μmoles free cholesterol $\times$ 387 $-$ μmoles cholesterol ester $\times$ 651 $-$ μmoles phospholipid $\times$ 760 $-$ μmoles free fatty acids $\times$ 282] $-$

II [μmoles total lipid $\times$ 285 $-$ μmoles free cholesterol $\times$ 387 $-$ μmoles cholesterol ester $\times$ 651 $-$ μmoles phospholipid $\times$ 760 $-$ μmoles free fatty acids $\times$ 282] 92/884 +

III [μmoles cholesterol ester $\times$ 651 + μmoles free cholesterol $\times$ 387 $-$ μmoles total cholesterol $\times$ 387] +

IV [μmoles free fatty acids $\times$ 282] +

V [μmoles phospholipid $\times$ 760 $-$ 0.475
μmoles phospholipid $\times$ 41 $-$ 0.475
μmoles phospholipid $\times$ 96 $-$ 0.475
μmoles phospholipid $\times$ 104 $-$ 0.475
μmoles phospholipid $\times$ 41 $-$ 0.475
μmoles phospholipid $\times$ 96 $-$ 0.475
μmoles phospholipid $\times$ 44 $-$ 0.05
μmoles phospholipid $\times$ 281 $-$ 0.05
μmoles phospholipid $\times$ 96 $-$ 0.05
μmoles phospholipid $\times$ 104]

I and II in the above equation give the contribution to total fatty acids by neutral glyceride fatty acid. The factor 92/884 is the ratio of the molecular weight of

glycerol to the molecular weight of an average triglyceride. Thus II refers to the contribution of glycerol in the Bragdon procedure and must be subtracted out. Therefore, I – II = micromoles of neutral glyceride fatty acid.

III gives the contribution of cholesterol ester fatty acid.

IV gives the contribution of free fatty acids.

V gives the contribution of phospholipid fatty acids. The 0.475 factors refer to the relative contributions of lecithin and cephalin; the factors 41, 96, 104, and 44 refer, respectively, to the molecular weight of glycerol less 3 OH, the molecular weight of the $-HPO_4$-moiety, the molecular weight of the choline base moiety less the OH esterified to $-HPO_4$-, and the molecular weight of ethanolamine less the OH esterified to $-HPO_4$-. The factor 0.05 refers to the assumed contribution of sphingomyelin; 281, to the molecular weight of the sphingosine moiety less the oxygen esterified to phosphorus; 96, to the molecular weight of the $-HPO_4$- moiety; and 104, to the molecular weight of the choline base moiety less the OH esterified to $-HPO_4$-.

Collecting all terms, the general equation simplifies to the following: μg of fatty acids = 255 μmoles total lipid − 145 μmoles phospholipid − 387 μmoles total cholesterol + 40 μmoles free cholesterol + 68 μmoles cholesterol ester + 29 μmoles free fatty acids.

Assuming that free fatty acids are negligible in liver and that the ratio of free cholesterol : cholesterol ester = 4,
μg of fatty acids = 255 μmoles total lipid − 145 μmoles phospholipid − 387 μmoles total cholesterol + 4/5 (40) μmoles total cholesterol + 1/5 (68) μmoles total cholesterol = 255 μmoles total lipid − 145 μmoles phospholipid − 341 μmoles total cholesterol.

The other equations in the text were derived in an analogous manner.

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Effect of Source and Level of Dietary Protein on Liver Enzyme Systems in the Young Pig [1]

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ABSTRACT Forty-eight young, rapidly growing, crossbred pigs were fed 8, 12, 16 or 20% protein starch-base diets, with the protein supplied by fish filet flour (FF), dried skim milk (DS) or soybean meal (SM), in an attempt to determine whether the activities of selected liver enzyme systems would reflect level and quality of dietary protein. The liver homogenates (1:5:liver:ice water) were assayed to determine the activities of xanthine oxidase (μliters O_2/hour/g wet liver), glutamic-pyruvic transaminase (mg pyruvic acid formed/30 minutes/g wet liver) and succinic dehydrogenase (mg formazan formed/30 minutes/g wet liver). Rate of gain and feed conversion efficiency were improved significantly as level of dietary protein was increased from 8 to 16%; liver weight and percentage nitrogen also increased significantly with increasing level of dietary protein. Liver xanthine oxidase activity was variable and did not generally reflect source or level of dietary protein. The activity of glutamic-pyruvic transaminase increased linearly with increasing levels of dietary protein from DS and SM. Succinic dehydrogenase activity increased linearly with increasing levels of dietary protein for all sources of protein. Liver succinic dehydrogenase activity was more closely related to average daily gain, liver weight and per cent liver nitrogen than was either xanthine oxidase or glutamic-pyruvic transaminase activity. Further, more elaborate studies are necessary to determine the usefulness of liver enzyme activities in evaluating protein quality for the young pig.

Researchers are continuously attempting to develop more precise means of evaluating the nutritive value of proteins. The protein efficiency ratio (1) and the biological value (2) methods continue to be used, and the biological value of proteins has been used as a reference in evaluating such methods as the chemical score (3) and the essential amino acid index (4). The studies of Williams et al. (5, 6) indicated the effects of methionine deficiency and of availability of amino acids on a specific body protein, xanthine oxidase. Litwack and co-workers (7–10) utilized xanthine oxidase activity as a measure of protein quality. They suggested that since xanthine oxidase activity measures the formation of that enzyme protein it should provide a suitable criterion for estimation of protein value. Furthermore, they reported the response of liver xanthine oxidase to be very sensitive to quality of protein. Muramatsu and Ashida (11–14) studied the responses of liver xanthine oxidase, glutamic-pyruvic transaminase and succinic dehydrogenase to source and level of dietary protein and to amino acid supplementation. They, too, demonstrated the sensitivities of liver enzymes to source and level of dietary protein and in some instances have shown a close relationship between liver enzyme activities and liver nitrogen as well as growth rate.

The objectives of the study reported here were 1) to determine the effects of 3 sources and 4 levels of dietary protein (table 1) on activities of selected liver enzymes in the young pig, and 2) to study the feasibility of using activities of liver enzyme systems to predict the nutritive value of dietary proteins for the young pig. The usefulness of liver enzyme activities in evaluating dietary protein has not been

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TABLE 1
Composition of experimental diets

Ingredients [1]	% protein											
	8	12	16	20	8	12	16	20	8	12	16	20
Cornstarch, g	5928	5502	5066	4640	4515	3367	2229	1084	5225	4443	3660	2877
Sugar, g	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000
Bleachable fancy tallow, g	300	300	300	300	300	300	300	300	300	300	300	300
Fish filet flour, g [2]	890	1330	1780	2220								
Skim milk, g [3]					2420	3640	4850	6060				
Soybean meal, g [4]									1600	2400	3200	4000
Dicalcium phosphate, g [5]	276	248	219	190	193	123	53	—	276	247	218	189
Calcium carbonate, g [5]	56	70	85	100	22	20	18	6	49	60	72	84
High zinc, trace mineral salt, g [6]	50	50	50	50	50	50	50	50	50	50	50	50
Vitamin-antibiotic premix, g [7]	500	500	500	500	500	500	500	500	500	500	500	500

[1] Air-dried basis.
[2] Dried, defatted fish filet flour; 90% protein (VioBin Corporation, Monticello, Illinois).
[3] Spray-dried skim milk; 34% protein.
[4] Soybean meal, solvent process; 50% protein.
[5] Dicalcium phosphate and calcium carbonate added in variable amounts as necessary to provide final levels of 0.8% calcium and 0.6% phosphorus.
[6] Provided 40 ppm supplemental zinc in the complete diet.
[7] Supplied per kg of diet: (in milligrams) riboflavin, 4.4; Ca pantothenate, 17.6; nicotinic acid, 26.4; thiamine·HCl, 6.6; pyridoxine·HCl, 3.3; choline chloride, 440; antibiotic, 275; (in micrograms) vitamin B_{12}, 11.0; and (in IU) vitamin A, 4400; vitamin D_2, 440; and *dl*-α-tocopheryl acetate, 220. The antibiotic consisted of a 2:2:1 mixture of chlortetracycline, sulfamethazine and procaine penicillin.

studied for the very young rapidly growing pig and it is not known whether results obtained with the laboratory rat are applicable to the young pig.

The 3 liver enzymes selected for study and the reasons for selection were: xanthine oxidase, because it has been shown to be very sensitive to amino acid deficiencies, protein depletion and low levels of dietary protein in the rat; glutamic-pyruvic transaminase, because the level of activity should reflect the utilization of amino acids for energy and the magnitude of amination of carbon skeletons to form amino acids; and succinic dehydrogenase, because the level of its activity should reflect energy breakdown in the body as associated with rapid growth and its accompanying metabolic activity.

EXPERIMENTAL

Forty-eight crossbred pigs produced by Duroc × Yorkshire dams and sired by Poland or Yorkshire males (one-half sired by each breed) and averaging 6.5 kg when weaned at an average age of 25 days were used. Replicate pairs of pigs (one male castrate and one female) were assigned at random to each treatment with the restriction that full-sibs would not be assigned to the same treatment. Within each replicate, each of the 12 pairs of pigs was randomly assigned to one of 12 adjacent pens which were 44 × 125 cm in size (2750 cm^2 floor space/pig) and equipped with slatted wooden floors. The pigs were individually weighed initially, at weekly intervals and at the termination of the experiment.

The pigs were placed on test when weaned and fed the assigned diets 3 times daily for 30-minute periods for the duration of the study. Semipurified diets based on cornstarch and containing 8, 12, 16 or 20% protein were fed for a 22-day period. Fish filet flour, dried skim milk and soybean meal were used as 3 sources of dietary protein (table 1). The composition of the experimental diets is shown in table 1. Pigs of the weight and age indicated were used because of the desire to use experimental subjects with maximal impetus for true growth.

To obtain liver samples, each pig was rendered unconscious, 4 hours post-feeding, by a sharp blow on the head; a mid-

line incision was made and the liver excised, trimmed free of connective tissue, blotted free of blood and weighed. The pigs were killed immediately by exsanguination. A 50-g portion of liver was then homogenized in an Osterizer blender with 5 volumes of water. The homogenate was filtered through 2 layers of surgical gauze and the filtrate divided into 3 separate aliquots which were frozen at −20° in polyethylene bottles. The remainder of the liver was sealed in a polyethylene container and frozen at −20°.

Prior to chemical analyses the liver samples were completely thawed and minced for one minute at low speed in an Osterizer blender. Nitrogen was determined by the conventional macro-Kjeldahl procedure. Ether extract was determined by the Mojonnier method (15).

Liver homogenates were thawed for 24 hours at 2° and thoroughly mixed prior to assaying for enzyme activity. Xanthine oxidase activity (μliters O_2/hour/g wet liver) was determined manometrically using a modification of the method of Muramatsu and Ashida (11) as follows: The main compartment of the Warburg reaction vessel contained 1 ml liver homogenate, 1 ml 0.03 M pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) buffer of pH 8.6 and 0.2 ml 0.01 M methylene blue. The side arm contained 0.8 ml 0.25% xanthine, in 0.06 M NaOH. The center well contained 0.2 ml 20% KOH. After a 35-minute period of incubation for temperature equilibration the substrate was tipped. Five minutes after the addition of the substrate the manometers were closed and readings taken every 20 minutes for one hour.

Glutamic-pyruvic transaminase activity (mg pyruvic acid formed/30 minutes/g wet liver) was determined by modifying the colorimetric method of Rosen et al. (16) as follows: Reagent A contained 120 ml 0.17 M 2-amino-2-methyl-1, 3-propandiol buffer of pH 8.9, 25 ml 0.5 M L-alanine, 15 ml 0.1 M α-ketoglutaric acid and 0.75 ml 10% bovine plasma albumin. L-alanine was replaced by water in reagent B which was used for blanks. After a 15-minute period of incubation, for temperature equilibration of 1 ml reagent A or B, 100 μliters liver homogenate were added to each tube. All tubes were incubated 30 minutes at 37°. Two drops of trichloroacetic acid (TCA) were added to each tube to terminate the reaction. One milliliter 0.1% 2, 4-dinitrophenylhydrazine (DNP) was then added, without necessity of removing the precipitated protein, and the reaction allowed to proceed for 5 minutes at 37° at the end of which time 2 ml water-saturated toluene were added and the mixture was shaken vigorously. The samples were then centrifuged to break any emulsion and 1 ml of the toluene layer removed and added to 6 ml 2.5% alcoholic KOH in a colorimeter tube. This mixture was shaken vigorously, allowed to stand for five minutes and again shaken vigorously to dissolve any turbidity. The color intensity of the solution was read in a Coleman Universal Model 14 spectrophotometer at 530 mμ. The amount of pyruvate formed was determined from a standard curve obtained using 25- to 250-μliter aliquots of 0.01 M solution of sodium pyruvate.

Succinic dehydrogenase activity (mg formazan formed/30 minutes/g wet liver) was determined by modifying the colorimetric method of Muramatsu and Ashida (11) as follows: A reaction tube containing 1 ml 0.1 M phosphate buffer and 0.5 ml 1 M succinic acid, in 1 M NaOH, was incubated for 15 minutes at 37°. Succinic acid was replaced by water in those tubes used as blanks. One-half milliliter 0.32% freshly prepared triphenyltetrazolium chloride (TPTZ) was added and the preparation incubated for 5 minutes at 37°. One milliliter liver homogenate was added and the reaction allowed to proceed for 30 minutes at 37°. The reaction was terminated by adding 10 ml *n*-butanol followed by vigorous shaking. The solution was then centrifuged to separate the butanol extract from the precipitated protein. The butanol extract was then transferred to a cuvette and the color intensity read in a Coleman Universal Model 14 spectrophotometer at 480 mμ. The amount of formazan formed was determined from a standard curve obtained by using 200- to 1000-μliter aliquots of a 0.2% solution of reduced TPTZ. The TPTZ was reduced by adding 100 mg sodium hydrosulfite ($Na_2S_2O_4$) to 100 ml freshly prepared 0.2% TPTZ.

RESULTS

The data for average daily gains and feed-to-gain ratios (table 2) indicate that the pigs were consuming adequate amounts of the dry diets to support suitable gains of pigs of the weight and age used in this study. Those pigs fed diets based on dried skim milk and containing 16 and 20% protein required 228 and 319 g protein, respectively, per kg gain and those pigs fed 16 and 20% protein diets based on soybean meal required 265 and 350 g protein per kg gain, respectively. Pigs fed diets based on dried skim milk gained significantly more rapidly ($P < 0.05$) than those fed diets based on fish filet flour (table 3). The performance of pigs fed diets based on soybean meal also tended to be superior to that of pigs fed fish flour diets. Level of dietary protein exerted a highly significant effect on rate of gain and there was a highly significant ($P < 0.01$) linear trend toward greater daily gains with increasing level of dietary protein; however, there was no increased growth response when the level of dietary protein was increased from 16 to 20% when either dried skim milk or soybean meal was the source of protein.

Feed-to-gain ratios were not significantly affected by source of dietary protein. However, highly significant differences resulted due to level of dietary protein and there was a significant linear decrease in the

TABLE 2

Influence of source and level of dietary protein on average daily gains and feed conversion efficiencies of young pigs [1]

Dietary protein	Protein source	Avg daily gain	Feed/gain
%		*g*	*kg*
8	Fish filet flour	73	3.64
12	Fish filet flour	103	3.01
16	Fish filet flour	128	2.32
20	Fish filet flour	214	1.58
8	Skim milk	130	2.53
12	Skim milk	195	2.02
16	Skim milk	277	1.43
20	Skim milk	245	1.59
8	Soybean meal	70	4.19
12	Soybean meal	144	2.35
16	Soybean meal	261	1.66
20	Soybean meal	250	1.75

[1] Four animals/treatment; average initial weight of 6.5 kg; feeding period of 22 days' duration.

TABLE 3

Results of variance analysis of the effect of source and level of dietary protein

Source of variation	df	Mean square						
		Avg daily gain	Feed/gain ratio	Liver wt	Liver nitrogen	Glutamic-pyruvic transaminase	Succinic dehydrogenase
Source of protein	2	27691.00*	0.6210	5957.58	6.7884*	126.4053**	3.5966*
Level of protein	3	55314.24**	2.0319**	33651.74**	15.3140**	17.9817	2.1910*
Linear	1	157132.84**	5.5754**	88742.60**	45.4314	50.7288*	6.5043**
Source × level	6	4583.97		1243.80	0.8476	7.3481	0.3382
Error	36	2236.94	0.1902	2426.83	0.3080	2.5413	0.2813

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

TABLE 4

Influence of source and level of dietary protein on weight and composition of livers of 6-week old pigs [1]

Protein	Protein source	Wet wt	Dry matter	Ether extract	Nitrogen
%		g	%	%	% fat-free dry matter
8	Fish filet flour	223	28.77	3.96	7.68
12	Fish filet flour	251	29.18	4.06	8.34
16	Fish filet flour	288	29.34	4.30	9.14
20	Fish filet flour	314	28.22	3.93	10.66
8	Skim milk	246	28.59	3.84	8.68
12	Skim milk	278	28.27	3.61	9.66
16	Skim milk	354	27.97	3.69	10.52
20	Skim milk	340	28.80	3.81	10.30
8	Soybean meal	199	29.66	3.91	8.48
12	Soybean meal	229	28.54	3.87	9.96
16	Soybean meal	340	28.84	3.86	10.96
20	Soybean meal	324	28.74	3.95	11.66

[1] Means for 4 pigs/treatment.

quantity of feed required per unit of gain with increasing protein content of the diets. This trend was expected in view of of the relatively high negative correlation ($r = -0.92$) between rate and efficiency of gain as well as reports of other workers (17–19).

Liver weight increased linearly as the level of dietary protein was increased (table 4). Source of protein, however, did not significantly affect liver weight. There was a highly significant correlation (fish flour, $r = 0.80$; skim milk, $r = 0.90$; soybean meal, $r = 0.90$) between average daily gain and liver weight irrespective of protein source. The percentage of nitrogen in the fat-free dry matter of the livers from pigs fed fish filet flour diets was significantly less ($P < 0.05$) than that in livers from pigs fed diets based on dried skim milk or soybean meal. There was a significant ($P < 0.01$) linear increase in the percentage of liver nitrogen as the level of dietary protein increased. A highly significant correlation existed between average daily gain and liver nitrogen (fish flour, $r = 0.68$; skim milk, $r = 0.76$; soybean meal, $r = 0.85$) irrespective of source of dietary protein.

Ether extract content of the livers was not significantly affected by either source or level of dietary protein (table 3). The dry matter content of the livers was fairly constant 28.74%.

Liver xanthine oxidase response, as measured by microliters of oxygen consumed per hour per gram of wet liver, was variable (fig. 1) and did not generally reflect the level of dietary protein. Xanthine oxidase activity was high in livers from those pigs fed 16% protein from soybean meal compared with the activity in livers

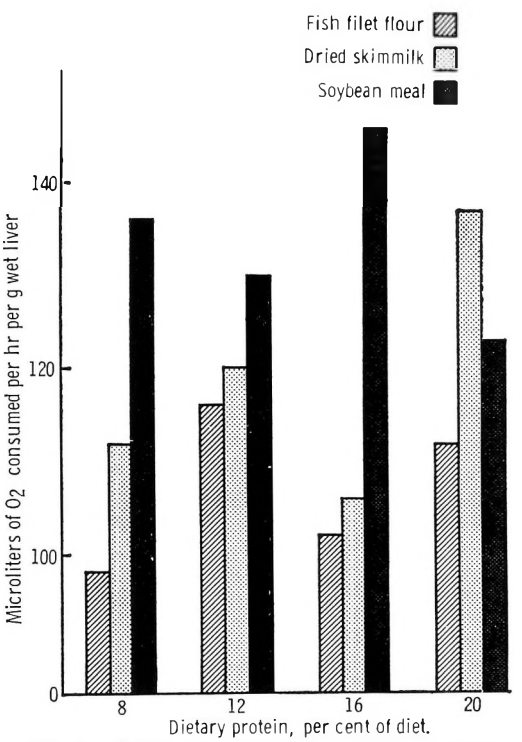

Fig. 1 Effect of source and level of dietary protein on liver xanthine oxidase activity (μliters of O_2 consumed/hour/g wet liver).

from pigs fed 16% protein diets based on dried skim milk or fish filet flour.

Liver glutamic-pyruvic transaminase activity was not affected by level of dietary protein when fish flour was the source of protein (fig. 2). The transaminase activity increased slightly as the level of dietary protein from dried skim milk was increased from 8 to 20%. When soybean meal was the source of dietary protein, an increase in level of dietary protein from 8 to 20% doubled the enzyme activity. There were significant correlations between glutamic-pyruvic transaminase activity and average daily gain, and liver nitrogen, when the pigs were fed soybean meal diets.

Succinic dehydrogenase activity in livers from pigs fed fish filet flour was significantly less ($P < 0.05$) than that in the livers from pigs fed dried skim milk diets and a highly significant difference ($P < 0.01$) existed between the means for fish filet flour diets and those based on soybean meal (fig. 3). There was a significant

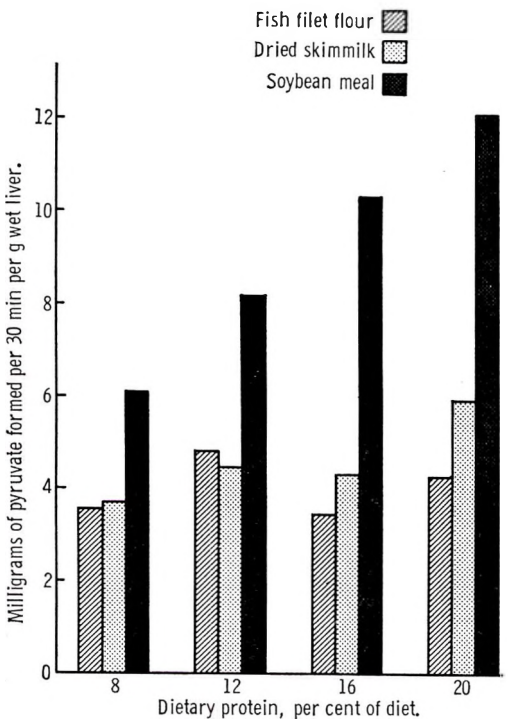

Fig. 2 Effect of source and level of dietary protein on liver glutamic-pyruvic transaminase activity (mg of pyruvate formed/30 min/g wet liver).

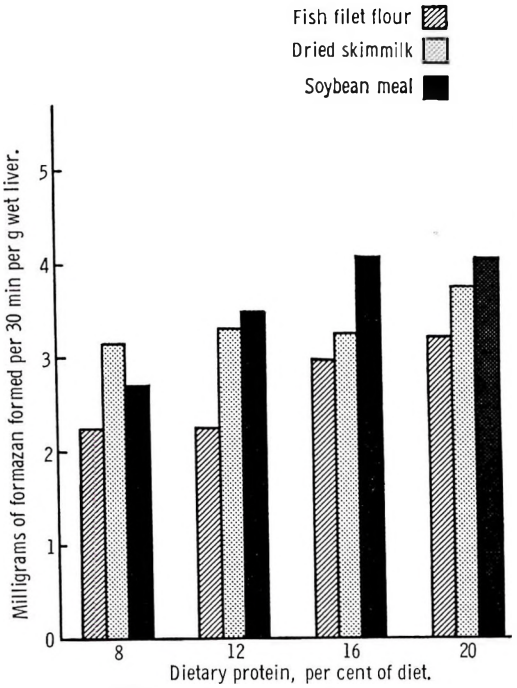

Fig. 3 Effect of source and level of dietary protein on liver succinic dehydrogenase activity (mg of formazan formed/30 min/g wet liver).

linear increase in dehydrogenase activity as the level of dietary protein was increased from 8 to 20%. A significant correlation ($r = 0.51$) existed between average daily gain and liver succinic dehydrogenase activity in those pigs fed fish filet flour. The correlation ($r = 0.68$) between average daily gain and succinic dehydrogenase activity of livers from pigs fed soybean meal was highly significant.

DISCUSSION

The lesser average daily gains exhibited by those pigs fed fish filet flour diets may have been due to tryptophan not being fully available from the fish filet flour. The addition of tryptophan to corn-fish meal diets has improved such diets for growing pigs (20, 21). The levels of amino acids contained in the diets based on the 3 different sources of protein did not differ markedly. Pigs fed diets based on dried skim milk or soybean meal showed the greatest increases in average daily gain between levels of 8 and 16% dietary protein, beyond which the gains did not appear to increase.

Feed-to-gain ratios, although based on short-term feeding periods as were the daily gains, were not affected significantly by the source of protein, perhaps because of the very poor feed-to-gain ratios of the pigs fed the 8% protein soybean meal diet.

The highly significant correlation between the average daily gain and liver weight, irrespective of protein source, was probably a direct result of those pigs fed the higher protein diets being larger. The highly significant linear increase in liver nitrogen, as the protein content of the diet was increased, was indicative of the increased intake of protein. Those pigs fed the higher protein diets may actually have been deaminating amino acids for use of the carbon skeletons as energy with the resultant accumulation of non-protein nitrogen in the liver. Also, an increased rate of protein synthesis would have been expected to occur in those animals which were growing at a more rapid rate and therefore more nitrogen would have been present in the liver.

Liver xanthine oxidase response did not generally reflect the level of dietary protein as had been reported previously by investigators using rats as experimental animals (8, 11–13). Under these experimental conditions it would not be feasible to use liver xanthine oxidase activity as a criterion for determining dietary protein requirement as has been suggested by Muramatsu and Ashida (12) for rats. Those workers indicated that xanthine oxidase activities, obtained from livers of rats fed low levels of dietary protein, did not show any regular pattern and also that the maximal value of the xanthine oxidase activity did not reflect protein quality.

Liver glutamic-pyruvic transaminase activity was more markedly affected when pigs were fed diets containing soybean meal as the source of protein. The increase in glutamic-pyruvic transaminase activity, as demonstrated in this study when pigs were fed the higher levels of protein from soybean meal, has been postulated by Rosen et al. (16) to be associated with an increase in gluconeogenesis.

Liver succinic dehydrogenase activity was greater in livers from pigs fed soybean meal than in livers from pigs fed either dried skim milk or fish filet flour. This phenomenon is consistent with the results of Muramatsu and Ashida (14), working with rats, as they reported that vegetable protein, such as soybean protein or wheat gluten, enhanced the activities of both xanthine oxidase and succinic dehydrogenase. These workers also reported less growth of rats fed low levels of vegetable protein than rats fed low levels of animal protein; this phenomenon prevailed in the work reported here as those pigs fed 8% protein dried skim milk diets gained more rapidly than those pigs fed 8% protein soybean meal diets. Animals fed fish filet flour diets gained less rapidly; however, this may have been due to an unavailability of amino acids as none of the sources of protein was supplemented with any amino acid.

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A Salt Mixture Supplying the National Research Council Estimates of the Mineral Requirements of the Rat [1]

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ABSTRACT In view of the criticisms of the compositions of the salt mixtures commonly used in rat diets, growth studies were carried out with a mineral mixture based on the requirements listed by the National Research Council (NRC). Addition of sulfate to this mixture improved growth and the protein efficiency ratio. All mineral requirements estimated by the NRC were adequate for maximal growth of male weanling rats. A close correlation was noted between estimated mineral requirements and amounts present in fat-free tissues of adult rats.

In protein quality tests in rats, comparing protein sources with high and low ash content, discrepancies were encountered and upon investigation, the Hubbell, Mendel and Wakeman (1) salt mixture component of the diets was found to be responsible. Phosphate addition to a diet containing lactalbumin of low mineral content and 4% salt mixture increased growth.

In a review and evaluation of mineral mixtures commonly used for rat diets, Williams and Briggs (2) emphasized that these mixtures often contain (a) less than the estimated requirements listed by the National Research Council (NRC) (3) for some minerals, (b) much higher than estimated requirements of others, (c) minerals for which no dietary requirement has been shown, and (d) unchelated pro-oxidant metals which promote diet rancidity. Besides the obvious possibility of growth restriction caused by mineral deficiencies, imbalances between sodium, chloride and potassium may interfere with growth of rats, as has been observed in chicks (4), and excess of calcium over phosphate may cause development of kidney and bladder stones (5).

In view of these criticisms (2) of available mixtures, a mixture was formulated to supply the NRC estimates of the mineral requirements of the rat. The effect of this mineral mixture on growth and protein efficiency ratio (PER) was investigated in rats fed diets of the type commonly used in tests of protein quality containing this mixture both with and without added sulfate.

EXPERIMENTAL

Composition of salt mixture. Table 1 (composition A) shows the composition of the salt mixture which contains the NRC requirements when 2.64 g are contained in 100 g of diet. Selenium for which the NRC requirement is estimated to be 4 μg/100 g of diet was not included. Except for Torula yeast diets, growth effects from selenium addition in diets containing adequate vitamin E have not been reported (6); moreover selenium toxicity has been noted at low dietary levels (7). Composition A included the citrates of all pro-oxidant metals as recommended by Fox and Mickelsen (8). Citrates of copper, zinc and manganese which are not commercially available, were prepared by reacting the carbonates with citric acid.

Methods. Groups of male weanling rats of the Sprague-Dawley strain, caged individually in a constant-temperature room, were fed diets with the general composition shown in table 2. Individual weight gains and group food consumption were measured. The sulfate content of diets was determined by the method of Toennies and Bakay (9).

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[1] A preliminary report of these observations was presented before the 49th Annual Meeting of the Federation of American Societies for Experimental Biology 1965 Federation Proc., 24: 373 (abstract).

TABLE 1

Composition of the 2 experimental salt mixtures supplying the NRC mineral requirements of the rat[1]

	A Without sulfate	B With sulfate
	g	*g*
$CaHPO_4$	693.0	735.0
K_2HPO_4	152.0	81.0
K_2SO_4	—	68.0
NaCl	31.0	30.6
$CaCO_3$	58.2	21.0
Na_2HPO_4	22.0	21.4
MgO	25.5	25.0
Trace metal mixture [2]	18.3	18.0
Total	1000.0	1000.0
Supplies/100 g of diet	2.64 [3]	2.69 [3]

[1] Preparation: To 150 ml of water in a tared 1-liter beaker add potassium iodide, ferric citrate and 50 g citric acid. Heat to dissolve. Mix zinc carbonate, manganese carbonate and copper carbonate. Add mixture gradually, with stirring, to hot ferric citrate solution. Allow to stand overnight and dry in oven at 100°. Determine weight of mixture and add citric acid to make 100 g. Grind to fine powder.

[2]

Constituents:	*g*
Ferric citrate (16.7% Fe)	31.0
Zinc carbonate (56% Zn)	4.5
Manganese carbonate (44.4% Mn)	23.4
Cupric carbonate (55.5% Cu)	1.85
Potassium iodide	0.04
Citric acid to make	100.0

[3] The NRC requirements (3) and amount in mg/100 g of diet supplied by this level of addition are: calcium, 600; phosphorus, 500; sodium, 50; potassium, 180; chlorine, 50; magnesium, 40; manganese, 5; iron, 2.5; zinc, 1.2; copper, 0.5; and iodine, 0.015. Diet B also supplies 100 mg of sulfate/100 g of diet.

TABLE 2

Composition of diets

	g/kg
Protein source	95–160
Salt mixture	9–40
Corn oil	100
Vitamin mixture [1]	1
Choline chloride	1
Cellulose [2]	50
Glucose [3]	to make 1000

[1] Vitamin content per gram: (in milligrams) thiamine·HCl, 6.5; riboflavin, 12.5; pyridoxine·HCl, 6; niacin, 75; Ca pantothenate, 40; 2-methylnaphthoquinone, 0.5; biotin, 0.5; folic acid, 2.0; ascorbic acid, 50; p-aminobenzoic acid, 100; inositol, 100; *dl*-α-tocopherol, 120; and vitamin B_{12}, 25 μg; vitamin A palmitate, 6000 IU; and vitamin D_2, 3000 IU.

[2] Solka Floc, Brown Company, Berlin, New Hampshire.

[3] Unless otherwise specified in text, Cerelose, Corn Products Company, Argo, Illinois.

RESULTS AND DISCUSSION

The effects of several salt mixtures on growth rate and PER are listed in table 3 experiment 1. Considerable difference in growth rate and PER occurred, and PER was not closely related to the rate of growth. For example, the group receiving the Hubbel, Mendel and Wakeman mixture (1) grew more but had a lower PER than the group receiving the USP XIII no. 2 mixture. The group fed the USP XIV salt mixture showed both the highest rate of gain and PER. Weight gain with the NRC mixture was comparable to that of the USP XIV mixture but the PER was definitely lower.

At the 4% level of addition, the USP XIV salt mixture supplied about 1.2 mg of sulfate/g of diet. To investigate the possibility that this sulfate was responsible for the higher growth rate and PER, the effect of adding potassium sulfate to the NRC salt mixture was tested. The results are shown in table 3, experiment 2. Addition of potassium sulfate supplying 1.2 and 2.4 mg of sulfate/g of diet resulted in a 10% increase in growth.

A salt mixture was then formulated which, when added to a diet in the proportion of 2.69%, supplied the NRC estimated requirements of all minerals and also 1 mg of sulfate/g of diet or 1.5 mg/g at the 4% level of addition (table 1), composition B. The growth effects of composition B were compared with the experimental salt mixture without sulfate and the USP XIV mixture. The data obtained are listed in table 3, experiments 3 and 4. Sulfate addition in each case slightly increased growth and PER.

In none of the 3 experiments in which the effect of sulfate addition was tested were the growth differences between groups statistically significant at the 5% level, although in two of the three tests the differences approached significance. In these tests, the sulfate content of the diet before mineral addition was about 0.2 mg/g. The increments in growth and PER caused by addition of sulfate to suboptimal protein diets low in sulfate were small but reproducible. Comparison of the NRC mixture with added sulfate with the USP XIV mixture (table 3, exps. 2 and 3) indicated that both mixtures produced a similar PER but that the NRC mixture caused a higher growth rate.

To further assess the effect of sulfate, a diet low in sulfate was prepared. Ammonium caseinate was dissolved in water, was dialyzed for 48 hours against cold, running tap water and twice for 24 hours against

TABLE 3

Effect of salt mixtures on growth of rats

Exp. no.	Group	Salt mixture [1]	Protein content (N × 6.25)	No. rats/ group	Days fed	Wt gain	Protein intake	Gain (g) / Protein (g)	*P* values (wt gain differences)
			%			g	g		
1	A	USP XIII no. 2 [2]	10 [3]	7	21	66.2 ± 2.9 [4]	28.5	2.31	
	B	HMW (1) [2]	10	7	21	72.7 ± 4.4	34.0	2.14	
	C	Wesson (10) [2]	10	7	21	72.7 ± 2.2	31.2	2.34	
	D	Jones-Foster (11) [2]	10	7	21	74.0 ± 3.4	32.8	2.36	
	E	USP XIV [2]	10	7	21	82.3 ± 4.4	31.3	2.62	
	F	A [5]	10	7	21	80.0 ± 3.5	34.2	2.34	
2	A	A [5]	9 [3]	8	29	95.0 ± 4.4	36.4	2.61	
	B	A [5] + 0.22 g K_2SO_4	9	8	29	105.5 ± 3.0	38.2	2.77	A, B < 0.1
	C	A [5] + 0.44 g K_2SO_4	9	8	29	104.8 ± 3.2	39.2	2.67	
	D	USP XIV	9	8	29	95.6 ± 2.2	35.3	2.70	B, D < 0.05
3	A	A [5]	9 [3]	8	29	100.9 ± 5.8	37.0	2.72	
	B	B [6]	9	8	29	104.6 ± 3.5	36.6	2.86	A, B = 0.55
	C	USP XIV	9	8	29	96.5 ± 3.4	34.8	2.73	B, C < 0.2
4	A	A [5]	9 [3]	9	30	109.2 ± 4.4	42.1	2.59	
	B	B [6]	9	9	30	118.0 ± 2.6	42.7	2.76	A, B = 0.1
5	A	A [5]	8.8 [7]	9	32	92.5 ± 4.3	39.3	2.35	
	B	B [6]	8.8	9	32	117.1 ± 4.8	40.2	2.91	A, B < 0.01
6	A	A [5]	6.7 [8]	9	25	69.5 ± 2.5	20.4	3.45	
	B	B [6]	6.7	9	25	78.4 ± 2.6	22.1	3.55	A, B < 0.05

[1] Animals received 4.0 g/100 g of diet.
[2] General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] High nitrogen casein, Sheffield Chemical Company, Norwich, New York.
[4] Means ± SE.
[5] Experimental salt mixture without sulfate (composition A, table 1).
[6] Experimental salt mixture containing sulfate (composition B, table 1).
[7] Ammonium caseinate, Sheffield Chemical Company.
[8] Lactalbumin, Sheffield Chemical Company.

10 vol of distilled water, and was then lyophilized. The powdered cellulose [2] of the diet was slurried in 0.01 N HCl, filtered in a Büchner funnel, washed with distilled water until free of chloride, and air-dried. Reagent-grade glucose was used. The sulfate content of the diet was 0.05 mg/g. Distilled drinking water was supplied during the test period. Data showing the effect of addition of sulfate to this diet are listed in table 3, experiment 5. The difference in growth was highly significant and the PER of the group fed sulfate was 24% higher. Significant differences in growth also occurred when the same procedure was followed using washed lactalbumin for the protein source of the diets (table 3, exp. 6).

Several previous reports have described growth effects of sulfate in monogastric animals. Almquist (12) reviewed the subject and listed data indicating that sulfate addition to chick diets increased growth and feed efficiency, presumably due to a reduction of the breakdown of sulfur-containing amino acids for synthesis of sulfate-containing body constituents such as mucoitin sulfate. In adult cats, dietary sulfate lowered or entirely eliminated the requirement for sulfur amino acids.[3] In rats, sulfates were observed to increase growth (13, 14), and to be incorporated into tissues (14).

The results obtained in earlier studies and in these tests indicate that with diets containing suboptimal levels of protein and low levels of sulfate, addition of sulfate results in increased growth and PER; therefore, a salt mixture for comparing protein quality should contain sufficient sulfate to allow for variations in the sulfate content of the protein sources tested.

Adequacy of salt mixture for growth. In all experiments reported below, the NRC salt mixture containing sulfate (composition B, table 1) was used. To determine whether the estimates of mineral requirements listed by the NRC (3) were adequate for all elements, rats were fed the basal diet containing different amounts of salt mixture in a diet with an adequate protein level. Results are shown in figure 1.

The group receiving the adequate protein level (13.4% lactalbumin) and 100%

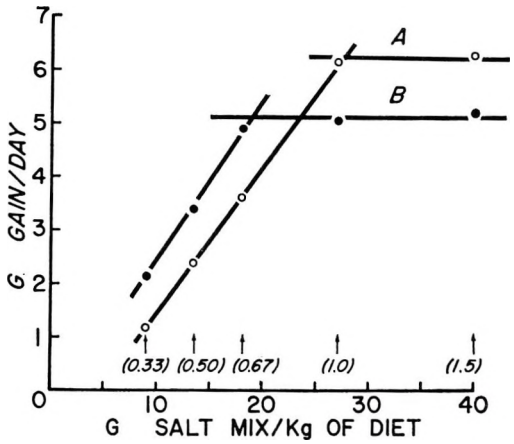

Fig. 1 Effect of various levels of addition of salt mixture (composition *B*, table 1) to the diet on the growth of male weanling rats. *A*, 13.4% lactalbumin diet fed for 21 days. The lactalbumin component of the diet supplied the following elements (mg/100 g of diet) in addition to the quantities supplied by the salt mixture: Ca, 0.8; P, 3.8; Na, 1.9; K, 0.8; and Cl, 1.7. *B*, 8.8% lactalbumin diet fed for 21 days. The lactalbumin component of the diet supplied the following elements (mg/100 g of diet) in addition to the quantities supplied by the salt mixture: Ca, 0.6; P, 1.8; Na, 1.9; K, 0.8; and Cl, 0.5. Values in parentheses indicate fractions of estimated requirements.

of estimated requirements for minerals grew at about the maximal rate for rats of the Sprague-Dawley strain fed semipurified diets (6.15 g/day) (15, 16). Rats receiving 150% of the estimated requirements grew at practically the same rate (6.25 g/day). These results indicate that the mineral requirements estimated by the NRC are adequate for maximal growth under these conditions. The close agreement between the estimate derived from the point of intersection of the lines with the theoretical value indicates that the estimate of the requirement for one or more of the minerals present is close to the actual amount required. Breuer et al. (16) used a salt mixture supplying the NRC requirements, but with a different composition; weight gains tended to be lower with casein diets containing this mixture, although the differences were not statistically significant.

[2] Solka Floc, Brown Company, Berlin, New Hampshire.

[3] Rambaut, P. C., and S. A. Miller 1965 Studies of sulfur amino acid nutrition in the adult cat. Federation Proc., 24: 373 (abstract).

The results obtained by adding different levels of salt mixture to a diet inadequate in protein (8.8% lactalbumin) are also shown in figure 1. In this case, the data indicate that a salt addition of 19 g/kg of diet (70% of estimated requirements) would have resulted in maximal growth. Comparison of the results of the 2 experiments shown in figure 1 indicates that less of the salt mixture is required when the growth rate is restricted by inadequate protein intake.

For routine use, addition of 40 g of salt mixture, supplying 150% of the estimated requirements of all minerals, is probably an adequate safeguard against possible increased requirements due to other dietary constituents. With diets containing amino acid hydrochlorides additional sodium may be required (17).

Tissue concentration of inorganic elements in relation to requirements. Bunge in 1902 (18) advanced the thesis that the mineral composition of the body resembled closely the mineral content of the milk of that species and hence that the minerals in milk are present in optimal proportion and concentration. Practically all salt mixtures recommended by subsequent investigators contained minerals in about the amounts present in milk (19).

As shown by the data in table 4, there is a direct correlation between the tissue concentrations and the NRC estimated growth requirements for minerals. The regression curve using these data is shown in figure 2. The data indicate that the amount of a mineral in 100 g of lean body mass multiplied by 0.58 yields a close approximation of the required amount per 100 g of diet or in the amount of diet supplying about 400 kcal. A relationship between tissue concentrations and requirements has also been shown in the rat, chick and pig between the essential amino acid content of fat-free tissue and amino acid requirement (20).

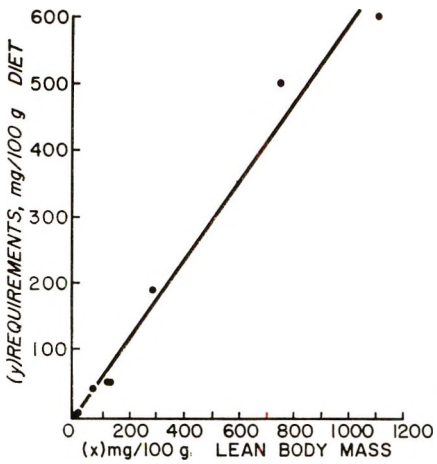

Fig. 2 Correlation between content of various minerals in the lean body mass of rats and the NRC (3) estimated requirements. The formula of the line, fitted by the method of least squares, is $y = 2.09 + 0.582\ x$. Correlation coefficient = 0.991. For identification of points, refer to table 4.

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TABLE 4

Comparison between mineral requirements/100 g of diet and the amounts present in the lean body mass of rats

Mineral	NRC requirement (3)	Content in lean body mass	Reference no.
	mg/100 g diet	*mg/100 g*	
Calcium	600	1110	(21)
Phosphorus	500	750	(22)
Potassium	180	287	(21)
Sodium	50	132	(21)
Chlorine	50	120	(21)
Magnesium	40	68	(21)
Iron	2.5	6.0	(22)
Zinc	1.2	3.0	(22)

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Amino Alcohols and Methyl Donors in Rats Fed Low Choline Diets Containing Added Cholesterol [1]

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ABSTRACT A study was carried out to elucidate the effects of ethanolamine and its N-methyl derivatives and of the methyl donors in weanling male rats consuming low choline diets containing added cholesterol for 7 days. The diets contained 36% casein. As the cholesterol level was increased in the diet the liver fat increased and there was an increase in the incidence and severity of hemorrhagic kidneys. The cholesterol effect was prevented by adding the N-methyl derivatives of ethanolamine but was only partially prevented by ethanolamine at the levels of ethanolamine studied. Methionine and betaine prevented the occurrence of kidney lesions but had little effect on the liver fat. Feeding the methyl donors and ethanolamine together in the diet containing added cholesterol was highly effective in preventing liver fat accumulation.

In the last few years we have been interested in choline antagonists and have reported on the effects of 2-amino-2-methylpropanol and 3-aminopropanol (1, 2). Both of these amino alcohols increased the symptoms of choline deficiency in young male rats. The N-methyl derivatives of ethanolamine, namely, monomethylethanolamine, dimethylethanolamine and choline, protected against these effects, whereas ethanolamine, betaine and methionine protected very little.

A great deal of evidence (3–9) has accumulated since 1933 indicating that cholesterol behaves as a choline antagonist in the rat and that choline protects against its antagonistic behavior. The present communication concerns the effects of the normally occurring amino alcohols and methyl donors in rats consuming choline-deficient diets containing added cholesterol. It can be concluded that cholesterol grossly behaves in choline-deficient rats in much the same way as 2-amino-2-methylpropanol and 3-aminopropanol.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain [2] were used in this study. Animals 19 to 21 days of age and weighing 40 to 55 g were placed in raised cages and fed experimental diets ad libitum for 7 days. Water was available to the animals at all times. Food consumption and body weights were recorded at intervals throughout the experimental period. At the end of the 7-day period the rats were decapitated and the kidneys examined grossly for hemorrhage.

For each set of feeding conditions in an experiment, 12 animals were used and were placed six to a cage into 2 cages. Usually each experiment consisted of from 48 to 96 rats distributed at random. Each liver fat determination was made on livers from 2 rats, thus 6 determinations per group of twelve. Usually the feeding of each dietary mixture to a group of 12 rats was carried out no less than 3 times.

The livers were removed, weighed, ground with anhydrous sodium sulfate and extracted with chloroform in a Soxhlet extractor for 4 hours. Following extraction, the chloroform extract was filtered through fat-free filter paper into tared flasks, concentrated to dryness, and the residue was dried to constant weight on a steam bath. The dry residue weight represents the liver fat as recorded in tables 1, 2 and 3.

The basal diet similar to the one used by Mulford and Griffith (10) consisted of dried brewer's yeast,[3] 6; agar, 2; salt mix-

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[2] Sprague-Dawley, Inc., Madison, Wisconsin.

[3] Anheuser-Busch, Inc., strain G.

ture,[4] 4; lard, 19.9; vitamins A and D supplement,[5] 0.1; calcium carbonate, 1.0; L-cystine, 0.3; casein,[6] 36; and sucrose 30.7%. Supplements of methyl donors and amino alcohols as well as cholesterol were first mixed with the lard and then mixed into the diets.

The 36% casein level in the diet was chosen because we wanted a basal low-choline diet that would, in the absence of added cholesterol, prevent the occurrence of hemorrhagic kidneys and also maintain the liver fat at close to the normal level for weanling rats. We also wanted the diet to permit adequate growth of the animals. Mulford and Griffith in 1942 (10) had shown that a minimum of 30% casein in the diet was able to accomplish these conditions. Their 18 to 42% casein diets were almost like the one used in this study. The main difference was in the casein level. Griffith in 1941 (11) fed a 40% casein diet containing 0.3% cystine with good growth of the rats. We (1) have made similar observations in rats consuming 42% casein diets.

RESULTS

Effect of cholesterol on the liver fat and kidney degeneration of young male rats. Table 1 shows that when cholesterol was incorporated into diets and fed to rats, the incidence of hemorrhagic kidneys and the level of liver fat were greater than in rats fed the basal diet. Increasing the amount of cholesterol in the diet led to higher levels of liver fat and an increase in the incidence of hemorrhagic kidneys.

Effect of added methyl donors and amino alcohols in diets containing added cholesterol. The results of a study on the effect of methyl donors and ethanolamine and its N-methyl derivatives in rats consuming the basal diet containing 33.6 μmoles of cholesterol/g of food (1.3%) are shown in table 2. Table 2 shows that the N-methyl derivatives of ethanolamine were markedly effective in reducing the level of liver fat due to the presence of added cholesterol in the diet. They were also effective in reducing the incidence of hemorrhagic kidneys. The data suggest that choline was somewhat more effective than dimethylethanolamine which in turn was somewhat more effective than monomethylethanolamine. The methyl donors, methionine and betaine, reduced the incidence of hemorrhagic kidneys but were not able to reduce the liver fat from the control level of 520 mg/liver (table 1). Ethanolamine was more effective than the methyl donors but was less effective than its N-methyl derivatives.

Table 3 shows the effect of ethanolamine in rats consuming the basal diet containing 33.6 μmoles of added cholesterol and either 36 or 54 μmoles of added betaine or methionine/g of food. Both the feeding of ethanolamine and betaine to-

[4] Salt mixture USP XIV; obtained from Nutritional Biochemicals Corporation, Cleveland.

[5] Contained in addition to vitamins A and D, vitamins C and B_6. It was incorporated into the diet such that the amounts of these vitamins added/100 g of diet were: vitamin A, 0.97 mg; vitamin D, 6.4 μg; vitamin C, 3.2 mg; and vitamin B_6, 0.63 mg; obtained as A.D.C. Drops from Parke, Davis and Company, Detroit, Michigan.

[6] Vitamin-Free Test Casein, Nutritional Biochemicals Corporation.

TABLE 1

Effect of cholesterol in rats consuming the low choline diet

Cholesterol/ g of food	No. of rats	Rats with hemorrhagic kidneys	Avg wt of liver fat	Liver fat	Avg daily wt increase	Avg daily food intake
μmoles		%	*mg*	*% of body wt*	*g*	*g*
0	36	0	225(4.0) [1]	0.27 ± 0.020 [2]	4.6(79) [3]	6.0
11.2(0.44) [4]	36	0	384(4.4)	0.50 ± 0.017	4.9(80)	6.1
22.4(0.86)	36	0	445(4.3)	0.56 ± 0.028	4.7(79)	5.9
33.6(1.3)	36	14	520(4.5)	0.66 ± 0.022	4.8(79)	6.2
56.0(2.2)	36	67	549(4.3)	0.75 ± 0.025	3.9(74)	5.7
112.0(4.4)	36 [5]	83	565(4.2)	0.82 ± 0.030	3.4(67)	5.6

[1] Numbers in parentheses represent average liver weight in grams.
[2] Mean ± SE.
[3] Numbers in parentheses represent average final body weight in grams.
[4] Numbers in parentheses represent % of added cholesterol in the diet.
[5] Six animals in this group died.

TABLE 2

Effect of amino alcohols and methyl donors on liver fat of young male rats consuming the low choline diet containing 33.6 μmoles of cholesterol/g of food

Supplement/ g of food	No. of rats	Avg wt of liver fat	Liver fat	Avg daily wt increase	Avg daily food intake
μmoles		*mg*	*% of body wt*	*g*	*g*
3.6 Ethanolamine	108(5) [1]	486(4.4) [2]	0.62 ± 0.028 [3]	4.4(78) [4]	6.1
7.2 Ethanolamine	60(2)	450(4.4)	0.56 ± 0.023	4.8(80)	6.3
14.4 Ethanolamine	36(0)	390(4.3)	0.48 ± 0.028	4.8(81)	6.8
33.6 Ethanolamine	12(0)	383(4.4)	0.50 ± 0.043	4.5(78)	6.2
3.6 MME [5]	96(2)	396(4.1)	0.52 ± 0.034	4.1(76)	5.6
7.2 MME	48(0)	256(4.2)	0.32 ± 0.015	4.9(80)	6.3
14.4 MME	36(0)	168(4.0)	0.21 ± 0.008	4.2(79)	6.3
3.6 DME [5]	84(0)	317(3.9)	0.42 ± 0.025	4.2(75)	5.6
7.2 DME	48(0)	236(4.2)	0.29 ± 0.011	5.1(81)	6.5
14.4 DME	36(0)	173(3.9)	0.21 ± 0.008	4.8(83)	6.7
3.6 Choline·Cl	72(0)	264(3.8)	0.35 ± 0.021	4.1(75)	5.7
7.2 Choline·Cl	48(0)	236(4.2)	0.28 ± 0.011	5.3(83)	6.9
14.4 Choline·Cl	36(0)	165(4.0)	0.21 ± 0.004	4.7(81)	6.6
36.0 Betaine·HCl	96(1)	526(4.3)	0.69 ± 0.070	4.1(75)	6.1
54.0 Betaine·HCl	24(0)	593(4.1)	0.78 ± 0.049	4.0(76)	5.8
72.0 Betaine·HCl	48(0)	504(4.5)	0.68 ± 0.032	3.6(73)	6.2
36.0 L-Methionine	96(5)	546(4.3)	0.74 ± 0.047	3.8(73)	5.6
54.0 L-Methionine	24(0)	474(3.8)	0.68 ± 0.028	3.2(70)	5.0

[1] Numbers in parentheses represent the percentage of animals having hemorrhagic kidneys.
[2] Numbers in parentheses represent average liver weight in grams.
[3] Mean ± SE.
[4] Numbers in parentheses represent average final body weight in grams.
[5] MME = monomethylethanolamine, DME = dimethylethanolamine.

TABLE 3

Effect of methionine and betaine on liver fat of young male rats consuming the low choline diet containing added ethanolamine and 33.6 μmoles of cholesterol/g of food

Ethanol-amine/ g of food	Methyl donor/ g of food	No. of rats	Avg wt of liver fat	Liver fat	Avg daily wt increase	Avg daily food intake
μmoles	*μmoles*		*mg*	*% of body wt*	*g*	*g*
3.6	36 L-Methionine	60 [1]	295(4.3) [2]	0.36 ± 0.013 [3]	4.8(81) [4]	6.2
7.2	36 L-Methionine	36	225(4.1)	0.28 ± 0.029	4.6(81)	6.4
14.4	36 L-Methionine	36	171(3.8)	0.22 ± 0.013	4.5(80)	6.1
3.6	54 L-Methionine	36	274(3.7)	0.36 ± 0.013	3.9(75)	5.6
7.2	54 L-Methionine	36	172(3.9)	0.22 ± 0.010	4.2(78)	5.8
14.4	54 L-Methionine	36	206(3.8)	0.26 ± 0.008	4.5(79)	6.0
3.6	36 Betaine·HCl	60	278(4.4)	0.33 ± 0.014	5.2(83)	6.6
7.2	36 Betaine·HCl	36	238(4.0)	0.28 ± 0.023	4.9(83)	6.9
14.4	36 Betaine·HCl	36	172(3.9)	0.22 ± 0.013	4.6(80)	6.3
3.6	54 Betaine·HCl	36	229(3.9)	0.29 ± 0.014	4.4(78)	6.2
7.2	54 Betaine·HCl	24	160(4.0)	0.20 ± 0.018	4.7(80)	6.2
14.4	54 Betaine·HCl	36	157(3.9)	0.20 ± 0.006	4.8(81)	6.3

[1] None of the animals shown in this table had hemorrhagic kidneys.
[2] Numbers in parentheses represent average liver weight in grams.
[3] Mean ± SE.
[4] Numbers in parentheses represent average final body weight in grams.

gether and the feeding of ethanolamine and methionine together were completely effective in reducing liver fat. In similar studies in which 2-amino-2-methylpropanol and 3-aminopropanol were antagonists, feeding of ethanolamine together with betaine was completely effective, whereas feeding ethanolamine together with methionine was only partially effective in reducing liver fat (1, 2).

DISCUSSION

The results presented here suggest that cholesterol as a choline antagonist behaved

very much like 2-amino-2-methylpropanol and 3-aminopropanol in the young male rat. It raised the level of liver fat and increased the severity of kidney hemorrhagic degeneration. The N-methyl derivatives of ethanolamine easily overcame cholesterol's anti-lipotropic effect, whereas the methyl donors, betaine and methionine, were unable to do so. Ethanolamine was moderately effective. Combinations of added ethanolamine and added methyl donors, however, were completely effective. The data suggest that cholesterol increases the requirement for the normally occurring amino alcohols.

It has been observed that 2-amino-2-methylpropanol in the diet inhibited the incorporation of ethanolamine and dimethylethanolamine into liver phospholipid (12). Choline incorporation was inhibited very little. 2-Amino-2-methylpropanol itself was incorporated into liver phospholipid (13), perhaps at the expense of the incorporation of the normally occurring amino alcohols. It has been suggested that ethanolamine and its N-methyl derivatives must first be incorporated into phospholipid before they are methylated to choline (14–16). It is postulated that cholesterol increases the severity of choline deficiency by inhibiting the incorporation of ethanolamine into liver phospholipid, thus preventing its methylation to phosphatidyl choline.

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Comparative Effect of Glucose Feeding on the Activities of Some Liver and Heart Enzymes in Rats [1]

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ABSTRACT Four enzymes involved in carbohydrate metabolism, as well as glutamic aspartic transaminase, and tissue glycogen were compared in livers and hearts of rats fed 60% glucose diets for 3, 7 and 14 days. These same activities were determined in control hearts, and isolated hearts perfused with 300 mg/100 ml glucose for 30 minutes. Whereas liver glycogen increased almost 40-fold by 7 days of diet, no significant change was found in heart glycogen. Heart glycogen decreased 24% during the preparation and preperfusion with Krebs buffer and continued to decrease during perfusion with 300 mg/100 ml glucose. Glucose feeding caused a marked rise in liver glucose-6-phosphate dehydrogenase which reached a peak at 3 days and thereafter declined slowly. No similar effect was noted in the heart enzyme which was initially one-third the level of that in liver. However, perfusion of isolated heart with glucose resulted in a significant decrease in glucose 6-phosphate dehydrogenase specific activity. Glucose feeding, or perfusion through heart, had little effect on the other measured liver or heart enzymes, except for heart glutamic-aspartic transaminase which initially declined during the first 7 days of diet and subsequently increased markedly.

The effect of feeding high carbohydrate diets on the induction or adaptation of various hepatic enzymes has been described by several investigators. Tepperman and Tepperman (1, 2) in 1958 reported that a 24-hour feeding of a high carbohydrate-low fat diet to fasted rats resulted in about a fourfold increase in the specific activity of the combined liver hexose monophosphate shunt enzymes, i.e., glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. By 48 hours of feeding, these activities had increased 17-fold over that of the controls (2). The authors suggested that the shunt dehydrogenases are inducible enzymes with glucose 6-phosphate as the probable inducer.

Fitch et al. (3) in 1959 extended these observations, showing that the activities of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were significantly increased by feeding either 60% glucose or fructose diets for 3 days. These workers also measured levels of phosphoglucomutase, glucose 6-phosphatase and phosphoglucoseisomerase activities after carbohydrate feeding.

Fitch et al. (4), in the same year, reported the measurement of 17 enzyme levels in control rats and rats fed for 7 days diets lacking free hexoses or diets containing 60% glucose or fructose. Hexose feeding resulted in significant increases in the activities of 10 enzymes, and the authors supported the concept (5) that increased traffic of a substrate through a specific metabolic pathway results in an increase in activities of enzymes involved in that pathway.

Glucose uptake by isolated perfused rat heart is affected by the dietary state of the animal from which the heart is obtained (6, 7). Also, glucose transport through the cell membrane is an important rate-limiting step for glucose utilization in heart tissue (8), and when transport is rapid, the rate of formation of glucose 6-phosphate may become rate-limiting in glucose metabolism (8). Shipp et al. (9), using glucose labeled either in C_1 or C_6, have reported that glycolysis appears to be the

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major metabolic pathway for glucose catabolism to carbon dioxide in the perfused rat heart, whereas the hexose monophosphate shunt activity is almost negligible.

Thus, based on the reports of metabolic adaptation of liver enzymes to high carbohydrate diets, an investigation was initiated to compare levels of certain liver and heart enzymes, and to determine their responses to high circulating glucose levels. In addition, the response of these same enzymes was studied in isolated rat hearts perfused with 300 mg/100 ml glucose solution.

EXPERIMENTAL

Treatment of animals. Adult male rats (200 to 250 g) of the Carworth Farms Wistar strain were maintained with standard laboratory ration [2] and water ad libitum until use. The animals were then caged individually, fasted overnight, and subsequently fed ad libitum for up to 14 days a purified diet containing 60% glucose. The composition of the diet is shown in table 1 and is comparable to that described by Hill et al. (10). Food and water consumption were recorded daily.

Enzyme and chemical analysis. Rats fed the laboratory ration (controls) or 60% carbohydrate diet for 3, 7 or 14 days (experimental groups) were killed after an overnight fast. Livers and hearts were rapidly removed, weighed and placed in ice-cold 0.05 M Tris-HCl buffer, pH 7.5 to 7.6, in a cold Potter-Elvehjem homogenizing tube. Tris buffer was added to give a 1:25 dilution of the original tissue, and the tissue was homogenized for 3 minutes in an ice bath using a Teflon pestle. This homogenate was then sonicated [3] for 3 minutes in a salt-ice bath to ensure complete removal of glycogen and enzymes. These procedures were checked for quantitative recovery of protein, glycogen and enzymes, comparing results against those using 0.2 M sucrose homogenization (3) and 0.2 M phosphate buffer, pH 7.4.

TABLE 1

Percentage composition of diet

	%
Glucose	60
Casein [1]	22
Purified cellulose [2]	9.8
Vitamin mixture [3]	0.2
Salt mixture [4]	5.0
Cod liver oil	3.0

[1] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.
[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[3] Hubbell et al. (11).
[4] Treadwell et al. (12).

A 4-ml aliquot was removed from the sonicated preparation for glycogen determination and the remainder was centrifuged at 4500 × *g* for 5 minutes in a Servall refrigerated centrifuge. This cell-free supernatant was used undiluted for determination of glucose 6-phosphate dehydrogenase; was diluted to 0.2 mg original tissue/ml for assay of lactic dehydrogenase and fructose-1,6-diphosphate aldolase; or was diluted to 0.05 mg original tissue/ml for determination of malic dehydrogenase (NAD) and glutamic-aspartic transaminase. All enzyme assays were performed by standard methods (13) and were checked against authentic substrates in Tris buffer.

For determination of hepatic and myocardial glycogen, a 4-ml aliquot of the sonicated preparation was added to 1 ml 5 N KOH and the mixture was placed in a boiling water bath for one hour. After cooling, 10 ml of 95% ethanol containing 0.1% lithium bromide were added and glycogen was allowed to precipitate overnight. The tube was centrifuged for 10 minutes at 2000 rev/min, and after decantation, the precipitated glycogen was washed with 5 ml 80% methanol (0.1% lithium bromide). Glycogen was recentrifuged, dissolved in 2 ml distilled water and determined using anthrone reagent (14). In the case of animals fed glucose diets, 0.2-ml aliquots of the KOH digest were used for glycogen precipitation.

Initially and after 3, 7 and 14 days of glucose feeding, glucose was determined on 10% trichloroacetic acid extracts of whole blood by the method of Longley and Roe (15). Liver and Heart protein were determined by the method of Lowry et al. (16).

Myocardial perfusion. For the myocardial perfusion study, ad libitum-fed rats were used. Hearts were removed from rats

[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[3] M.S.E. ultrasonic disintegrator, Instrumentation Associates, Inc., New York.

anesthetized with pentobarbital sodium [4] and placed in 37° Krebs-Ringer bicarbonate buffer pH, 7.4 (17). The technique for myocardial perfusion in a continuously recirculating system is described elsewhere (7). Coronary perfusion was carried out in control hearts with Krebs bicarbonate buffer for 5 minutes, and in experimental hearts with 300 mg/100 ml glucose in bicarbonate buffer for 30 minutes under aerobic conditions. Uptake of glucose was determined at 10-minute intervals, and following perfusion, the hearts were removed from the apparatus, weighed and treated as described above.

Apparent differences between enzyme levels were analyzed for significance by the *t*-test (18).

RESULTS

Tissue glycogen. Comparative data for levels of tissue glycogen and various enzymes, and the effects of feeding 60% glucose to rats for up to 14 days are shown graphically in figures 1–3, and are summarized in table 2. As shown in figure 1, feeding 60% glucose to rats ad libitum did not cause any significant increase in blood glucose during the first 7 days, but by 14 days blood glucose levels had doubled to 200 mg/100 ml. During this same period, liver glycogen increased 30-fold during the first 3 days, almost 40-fold by 7 days (table 2), and thereafter was relatively constant. Heart glycogen was about twice the level of liver glycogen in fasted rats, and although glucose feeding caused an apparent decrease in heart glycogen, this decrease was insignificant in the present study. Evans (21) in 1934 reported that myocardial glycogen was higher in 24-hour-fasted rats than in fed animals. However, Lackey et al. (22) in 1946 reported a significant increase in myocardial glycogen only after 72 hours' starvation. Thus, in general, glycogen in heart is variably higher in fasted rats than in fed animals and these levels are not significantly altered by feeding 60% glucose diet for 14 days.

Glucose 6-phosphate dehydrogenase. Glucose feeding caused a marked increase in the liver glucose 6-phosphate dehydrogenase level over the level in fasting rats (fig. 2 and table 2) which reached a peak at 3 days of feeding and declined slowly thereafter. This is the same effect reported by Tepperman and Tepperman (1, 2) and by Fitch et al. (3, 4), although the elevation of this enzyme was somewhat less than that reported previously. These data are consistent with the concept of temporary adaptation of hexose monophosphate shunt enzymes of liver in response to increased metabolic flow via this pathway

[4] Nembutal, Abbott Laboratories, Inc., North Chicago.

TABLE 2

Effect of high glucose diet on hepatic and myocardial glycogen and enzyme activities [1]

Tissue component	Fasted overnight		60% Glucose, 14 days	
	Liver	Heart	Liver	Heart
Protein, % of tissue	20.2 ± 0.3 [2]	13.5 ± 0.4 [3]	19.0 ± 0.2 [2]	13.6 ± 0.2 [2]
Glycogen, mg/100 g	234 ±32	432 ±44	9132 ±198*(7)	280 ±48
Glucose 6-phosphate dehydrogenase, units/mg protein	14.3 ± 1.2	4.4 ± 0.3	59.3 ± 8.4*(3)	3.3 ± 0.3
Malic dehydrogenase, units $\times 10^{-3}$/mg protein	11.1 ± 0.7	20.8 ± 1.3	5.1 ± 1.4*	17.4 ± 3.6
Aldolase, units/mg protein	181 ±12	236 ±21	175 ± 30	254 ±45
Lactic dehydrogenase, units $\times 10^{-3}$/mg protein	6.3 ± 0.5	9.3 ± 0.4	7.0 ± 0.6	9.3 ± 0.2
Glutamic-aspartic transaminase, units $\times 10^{-3}$/mg protein	6.3 ± 1.5	10.4 ± 2.9	4.2 ± 0.4	14.7 ± 1.5

[1] All enzyme values are expressed as Wroblewski units (19) except fructose-1,6-diphosphate aldolase which is in Bruns units (20).
[2] Each value is the mean of 4 animals ± SE.
[3] Each value is the mean of 8 animals ± SE.
* Significant at 5% level. Figures in parentheses indicate the days after feeding glucose.

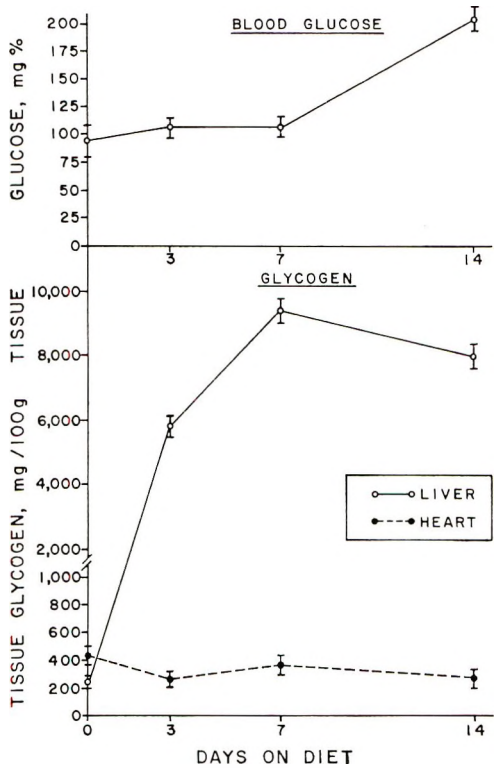

Fig. 1 Changes in blood glucose and liver and heart glycogen in rats fed 60% glucose diet. Values represent means ± SE.

(1, 4). The peak in activity at 3 days with subsequent reduction of activity by 14 days is comparable to the results reported by Tepperman and Tepperman in 1958 (2).

The level of glucose 6-phosphate dehydrogenase in heart was about one-third the level observed in liver as reported previously by Glock and McLean (23). There was no difference between this enzyme level in hearts of fasted or fed rats, and 60% glucose feeding for 14 days resulted in an insignificant change.

Malic dehydrogenase (NAD). The level of liver malic dehydrogenase in fasted rats was about half that in the heart. Although 60% glucose feeding did not alter the level of this enzyme in heart significantly over the 14-day period, there was a 50% decrease in liver malic dehydrogenase after the diet had been fed for 14 days. Fitch and Chaikoff (4) reported that 60% glucose feeding to rats for 7 days caused no significant changes in any of the measured tricarboxylic acid cycle enzymes except for the NADP- and NAD-dependent malic dehydrogenases which were increased about five-fold and 1.5-fold, respectively. Also, as these authors point out, feeding 60% glucose does not alter the hepatic conversion of acetate to carbon dioxide (presumably via the tricarboxylic acid cycle). These data were explained as reflecting increased conversion of pyruvate from glycolysis to oxalacetate and finally to aspartate. The observations of increased glutamic-aspartic transaminase activity supported the authors' views (4). However, in the present study, no increase in either liver malic dehydrogenase or glutamic-aspartic transaminase was noted (fig. 3) during the 14-day feeding regimen. These data would be consistent with hyperlipogenesis and an increased flow through the glucose 6-phosphate dehydrogenase system, with the energy and NADPH being utilized for fat synthesis (2) and a corresponding reduced flow through the TCA cycle. A study of the level of liver fatty acid-synthesizing enzymes would be of interest and might serve to clarify this point.

Fructose-1,6-diphosphate aldolase, lactic dehydrogenase and glutamic-aspartic transaminase. The level of heart aldolase appeared somewhat higher than that of liver

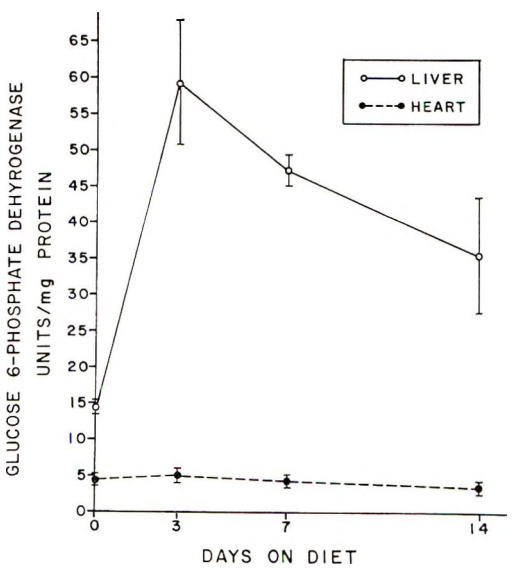

Fig. 2 Effect of 60% glucose diet on liver and heart glucose 6-phosphate dehydrogenase. Values represent means ± SE.

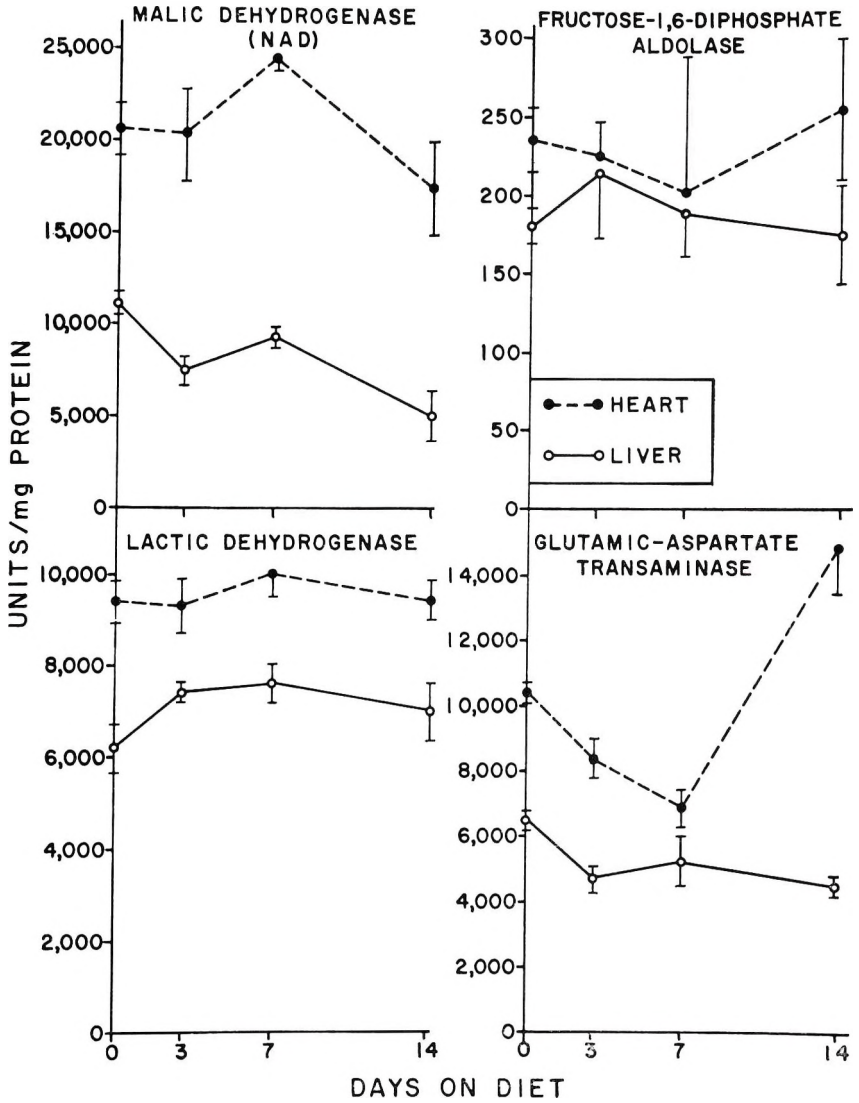

Fig. 3 Effect of 60% glucose diet on liver and heart malic dehydrogenase (NAD), fructose-1,6-diphosphate aldolase, lactic dehydrogenase and glutamic-aspartic transaminase. Values represent means ± SE.

but the difference was insignificant. Glucose feeding failed to cause significant changes in aldolase specific activities in either tissue during the experimental period. These data are consistent with those of Fitch and Chaikoff (4). However, these data cannot be interpreted to indicate no change in throughput via the glycolytic pathway since it has been shown that other glycolytic enzymes, such as phosphoglucose isomerase, phosphoglyceraldehyde dehydrogenase and phosphoglycerate kinase were increased 24 to 29% by 7-day glucose feeding (4).

Heart lactic dehydrogenase specific activity was about 50% higher than that of liver (table 2) and these levels were not significantly altered by glucose feeding. These results together with aldolase specific activities suggest that if there is in-

creased traffic via the glycolytic pathway due to glucose feeding, the altered rates were not sufficient to result in adaptation of these 2 enzymes during the experimental period. It is, however, possible that a transient enzyme adaptation occurred, as with glucose 6-phosphate dehydrogenase, and that the sampling times of 3, 7, and 14 days were inappropriate for detecting these increases. Comparison of the results of Fitch and Chaikoff (4) with the present data also suggest this latter possibility.

Glutamic-aspartic transaminase levels were similar in both liver and heart in fasted rats. Glucose feeding caused no significant change in this enzyme in liver over 14 days. However, in heart there was a progressive decrease during the first 7 days of diet, followed by a dramatic increase by the fourteenth day.

Isolated heart perfusion. Glucose uptake and oxidation to $^{14}CO_2$ by rat hearts perfused with 300 mg/100 ml glucose-U-^{14}C for 30 minutes is shown in table 3. Of the 250 μmoles of glucose-U-^{14}C available to the heart, there was about 20% uptake in 30 minutes, and, of the glucose extracted, 11% (5.5 μmoles glucose equivalents) were collected as $^{14}CO_2$ during the perfusion period. These data do not represent true oxidation to carbon dioxide since there is a 10 to 15-minute lag in $^{14}CO_2$ production before the rate becomes linear with time (24).[5]

The effect of glucose perfusion on myocardial glycogen and enzymes is summarized in table 4. As in the case of the 60% glucose feeding study, the hearts exposed to high circulating glucose showed a variable decrease in tissue glycogen compared with unperfused hearts. Some of this loss occurred during preperfusion with glucose-free buffer, as has been shown previously (25). Perfusion of hearts with 300 mg/100 ml glucose caused no significant

[5] Unpublished observations.

TABLE 3

Glucose uptake and oxidation by isolated rat hearts perfused with 300 mg/100 ml glucose for 30 minutes [1]

Heart wt	Glucose uptake		Glucose to $^{14}CO_2$[2]
g	*%*	*μmoles/g/30 min*	
1.241 ± 0.033 [3]	23.0 ± 0.3	50.0 ± 1.9	5.5 ± 1.1

[1] Conditions for the perfusion are described in the text and (7).
[2] Expressed as μmoles glucose equivalents/g heart/30 minutes.
[3] Mean ± SE.

TABLE 4

Effect of 300 mg/100 ml glucose perfusion on enzymes of rat heart

Myocardial component [1]	Fasted	Fasted; perfused with K–R bicarbonate buffer [2] 2½ min	Fasted; perfused with 300 mg/100 ml glucose, 30 min
Protein, %	13.5 ± 0.4 [3]	11.9 ± 0.3	11.0 ± 0.3
Glycogen, mg/100 g tissue	432 ±44	328 ±26	252 ±45
Glucose 6-phosphate dehydrogenase, units/mg/protein	4.4 ± 0.3	3.09 ± 0.4	1.85 ± 0.2
Malic dehydrogenase, units × 10^{-3}/mg protein	20.9 ± 1.3	17.7 ± 0.7	16.5 ± 1.5
Aldolase, units/mg protein	236 ±21	257 ±22	275 ±32
Lactic dehydrogenase, units × 10^{-3}/mg protein	9.3 ± 0.4	8.2 ± 0.2	7.3 ± 0.2
Glutamic-aspartate transaminase, units × 10^{-3}/mg protein	10.5 ± 2.9	7.6 ± 0.4	11.5 ± 3.9

[1] All enzyme values are expressed as Wroblewski units (19) except fructose-1,6-diphosphate aldolase which is in Bruns units (20).
[2] Krebs-Ringer bicarbonate buffer, pH 7.4 (17).
[3] Mean ± SE.

changes in the levels of malic dehydrogenase (NAD), aldolase, lactic dehydrogenase or glutamic-aspartic transaminase, but there was a significant decrease in the specific activity of glucose 6-phosphate dehydrogenase. The presence of the low level of this enzyme in heart initially, and its response to glucose infusion are in accord with the concept that the hexose monophosphate shunt pathway in heart muscle is of little significance as an energy pathway in myocardial tissue (9).

DISCUSSION

In a direct comparison of enzyme specific activities between hepatic and myocardial tissue, the levels of enzymes involved in glycolysis in heart tissue were 1.5 to 2 times the levels observed in liver, whereas in the fasted rat, the level of glucose 6-phosphate dehydrogenase in heart is one-third that in liver. These differences may be interpreted as reflecting relative levels of total glycolytic activity between the 2 tissues, since it has been postulated that the level of enzyme activity can be related to the metabolic activity of a pathway, and that this involves all enzymes of the pathway rather than only the rate-limiting enzyme or enzymes (4). In the present study, feeding the 60% glucose diet to previously fasted rats resulted in a marked increase in hepatic glucose 6-phosphate dehydrogenase activity, confirming previous reports (1–4), and the rate of increase and decline of this enzyme activity was comparable to that reported earlier by Tepperman and Tepperman (2). This increase can be interpreted as reflecting increased activity of the hexose monophosphate shunt pathway to accommodate the high levels of circulating glucose following glucose feeding. The level of glucose 6-phosphate dehydrogenase in heart, however, was unaltered during glucose feeding for 14 days, and in isolated hearts perfused with glucose, dehydrogenase activity was significantly lower than in control hearts. The initial low level of glucose 6-phosphate dehydrogenase activity in heart and the lack of inducibility of this activity is consistent with the previously suggested concept of the unimportance of the hexose-monophosphate shunt pathway in heart muscle (9). Thus, the majority if not all of the glucose utilized by heart during feeding or perfusion is metabolized by the already highly active pathway of glycolysis, since during these periods there is also net glycogenolysis. In further support of the concept that the glycolytic pathway in heart is at maximal or near maximal activity is the observation that feeding glucose to rats did not alter the levels of myocardial aldolase and lactic dehydrogenase, nor the level of the oxidative enzyme, malic dehydrogenase (NAD).

The significant elevation of transaminase activity in heart following 14 days of glucose feeding may reflect the nitrogen-sparing effect of glucose as discussed by Fitch and Chaikoff (4).

The primary difference between the present study and that reported previously (4) involves the enzymes of glycolysis and oxidation in liver. It had been reported that in rats fed laboratory ration, feeding 60% glucose diet for 7 days resulted in approximately 25 to 65% increases in the activities of 4 enzymes of glycolysis, as well as lactic dehydrogenase, whereas there was no change in fructose-1,6-diphosphate aldolase. Of the oxidative enzymes measured, only malic dehydrogenase (NAD) showed a significant increase (4). Since there is no increase in the rate of acetate oxidation in livers of glucose-fed animals, increases in oxidative enzyme activities should not have been expected. In the present study, control enzyme levels were obtained from fasted animals rather than rats fed the laboratory ration, and all animals were fasted overnight prior to killing. This difference itself might provide an explanation as to the lack of effect of 60% glucose feeding reported earlier (4), since in one-day-fasted animals, hepatic glycogenolysis would provide substrates for glycolysis and oxidation. Thus, there was no adaptive increase in levels of aldolase, lactic dehydrogenase or NAD following glucose feeding. The significant decrease in hepatic malic dehydrogenase after 14 days of diet might conversely represent: a) decreased ability of the liver to effectively oxidize acetate to CO_2; b) decreased conversion of α-keto acids to amino acids; or c) increased lipogenesis involving excess utilization of NADPH produced by the shunt pathway.

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Effect of Acute Starvation and Refeeding on Body Composition of Rats Fed Previously at Different Levels of Dietary Protein [1,2]

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ABSTRACT The effect of acute starvation and refeeding on body composition was studied in rats receiving high and low protein diets. For this purpose, weanling Sprague-Dawley male rats were fed a high protein diet (20% casein) or a low protein diet (5% casein) during an 8-week period, at the end of which the animals were starved for 2, 4, and 6 days. Rats in both dietary groups were treated similarly and after 6 days of starvation, each group was refed for 8 days with 5% or 20% casein diets. Body composition was somewhat similar at both protein levels. During starvation, the 2 groups of rats used their body fat reserves efficiently, although the malnourished animals utilized them at a more rapid rate. Higher body protein content was observed in all animals receiving the 20% casein diet. Although some differences among the groups studied are evident when the comparison is made in terms of average percentages, these differences are not as great as expected if the level of protein in the diet, the age of the animals, the length of time fed the experimental diets and the stress of acute starvation and refeeding are considered. When total body water, protein, and ash were related to fat-free weight, highly significant correlations were obtained. This information suggests, therefore, that the composition of the lean body remains relatively constant throughout all the experimental conditions, and depends on body size in growing animals.

The effect of acute starvation in the rat at different levels of protein nutrition has been studied in relation to serum lipids (1) and serum proteins (2). These observations have shown that the status of protein nutrition in the rat produces a disparity in the course of response of serum components to acute starvation.

Direct chemical analysis of experimental animals under a variety of conditions of growth and suboptimal nutrient intake have indicated a remarkable constancy of body composition (3–6). [3] It has been shown, for instance, that protein nutrition had little or no effect on percentage body composition, and that total body proteins appear to be weight-dependent (6).

Although there are some questions in extrapolating the results from experimental animals to humans, the basic information which supported the derivation of many of the equations used in calculating body composition by indirect methods, was obtained from laboratory animals.

The constancy of the composition of the lean body mass has been an accepted assumption in most of the body composition work. The concept of "chemical maturity" has been the basis of the different indirect methods involving the lean body mass (7).

Although the chemical composition of different animals under various dietary conditions has been studied, there is no information on the changes in body components of protein-malnourished animals subjected to the stress of acute starvation nor on the effect of refeeding after starvation.

The experiments to be described were designed to test whether the composition of the lean body mass remains relatively constant in rats at different levels of protein nutrition when they are submitted to acute starvation and refeeding with diets containing 5 or 20% casein.

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[2] INCAP Publication I-363.

[3] Wallace, W. M. 1959 Nitrogen content of the body and its relation to retention and loss of nitrogen. Federation Proc., *18:* 1125 (abstract).

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. B. Hart. J. Biol. Chem., *138:* 459, 1941.

[2] Alphacel, Nuritional Biochemicals Corporation, Cleveland.

[3] One hundred grams 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; distilled water to make 1 liter.

The experimental design was described in a previous report (2). The composition of the diets is shown in table 1. At the end of 8 weeks of feeding the experimental diets, 2 levels of protein nutrition were obtained: one group which had consumed the 5% casein diet and the other the 20% casein diet. As presented, each dietary group was divided into 6 subgroups of the same body weight. These groups were treated in the following way: subgroups 1, 2, 3, and 4 within each level of protein nutrition were starved for zero, 2, 4, and 6 days, respectively. Subgroups 5 and 6 were starved for 6 days and then refed for 8 days. Subgroups 5 within each dietary group were refed the low protein diet, while subgroups 6 were given the diet containing 20% casein. The animals were placed in individual all-wire screen cages with raised screen bottoms and were fed ad libitum. Water was available at all times. The rats were decapitated at the end of each experimental period, bled and the carcass was opened and carefully eviscerated.[4] The weight of the eviscerated animal was recorded.

The carcass and head were frozen, chopped and then ground in a meat grinder. This preparation was ground twice more in order to obtain a homogeneous sample. Aliquots of the samples were taken for water content determination. The dried samples were ground further in a laboratory-type Wiley mill and dried again to constant weight.

All methods used in determining the proximal composition were those given by the AOAC (8). Water content was determined in a vacuum oven at 70°. Fat was determined by continuous ether extraction in a micro-Soxhlet apparatus. Total nitrogen was obtained by the micro-Kjeldahl method using the modification of Hamilton and Simpson (9). Total proteins were calculated by the factor 6.25. The ash content was determined by calcination in a muffle furnace.

Analysis of variance was applied in the statistical study of the results. The multiple range test of Duncan (10) was used in the comparison of individual means. These statistical methods, and others used in the evaluation of the results, are those given by Snedecor (11).

RESULTS

The statistical comparisons shown in the following tables are those found within each dietary group. The comparisons between groups are not given in the tables.

In table 2, the body composition of the animals is expressed as the percentage of fresh tissue. No significant difference in initial pre-starvation values in water and fat content were observed (subgroups 1). Protein percentage was higher and ash lower in the group fed the 20% casein diet.

During starvation, the low protein group had an increase in water and protein, a decrease in fat, reaching very low values, and no significant changes in ash concentration. The high protein group, however, did not show any significant change in the percentage of the different body components. At the end of starvation, water and ash percentages were higher and fat lower in the 5% casein group.

During refeeding there was no significant change in water concentration when animals of both dietary groups were refed either of the diets. The water content of the animals in the low protein group, however, when refed the diet containing 20% casein after starvation, (5% casein group, subgroup 6), was higher than that of the other dietary group when refed the same

[4] The carcass was completely emptied of all viscera. Blood was not included in the body materials analyzed.

TABLE 2

Effect of acute starvation and refeeding on body composition of rats previously fed different levels of dietary protein [1]

Sub group	No. days starved	Casein refed post-starvation	Carcass wt		Water		Fat		Protein		Ash	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
		%	*g*		*% wet wt*		*% wet wt*		*% wet wt*		*% wet wt*	
					5% casein group							
1	0	—	82	20	59.07	1.44	14.99	2.17	19.68	1.13	4.24	0.76
2	2	—	72	16	59.67	3.21	12.70	4.23	21.25	1.01*	4.28	0.32
3	4	—	69	15	60.83	1.44	10.06	1.89**	20.82	1.06	4.58	0.33
4	6	—	61	10	64.92	1.80**	5.83	2.11**	21.91	1.04**	4.64	0.41
5	6	5	73	15	63.68	1.80	11.35	1.52**	18.86	0.75**	4.42	0.33
6	6	20	106	9	64.84	1.00	12.35	1.35**	18.21	0.78**	3.56	0.29**
					20% casein group							
1	0	—	267	35	61.43	2.85	12.40	3.63	22.26	1.27	3.20	0.31
2	2	—	250	34	63.37	1.96	9.60	2.14	21.74	1.07	3.09	0.62
3	4	—	234	32	59.79	4.08	11.72	4.44	21.96	1.10	3.42	0.20
4	6	—	231	27	61.48	2.70	9.52	2.55	23.07	1.30	3.40	0.37
5	6	5	250	32	61.32	1.69	13.23	2.11*	21.53	0.84*	3.64	0.41
6	6	20	265	30	61.47	2.84	12.75	3.35*	20.71	1.23**	3.19	0.27

* Significant difference at $P < 0.05$, and ** significant at $P < 0.01$, 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] $N = 6$ in all subgroups, except in subgroup no. 6 of the 5% casein group, where $N = 4$.

20% casein diet (20% casein group, subgroup 6).

The fat concentration increased significantly during refeeding, although the increase in the group previously fed the low protein diet was more pronounced. This group was also the one which during starvation had had the most drastic decrease in fat content.

The protein concentration during refeeding decreased in both dietary groups. The groups previously fed the low protein diet showed lower protein percentages than the other dietary group when they were refed either of the diets.

Finally, the ash content decreased significantly only in the low protein group when refed the 20% casein diet.

From these results, it appears that the main effect produced during starvation and refeeding is the disproportional change in the fat content that makes the other components vary in their percentage concentration.

If instead of comparing group percentages, the individual total weight body components are related to the fat-free carcass weight (FFW), then highly significant correlations are obtained. These relationships are shown in the following regression equations:

$$\text{Total water} = 0.29 + 0.6942\ (\text{FFW})$$
$$r = 0.9986;\ s_{yx} = 2.963.$$
$$\text{Total protein} = -1.81 + 0.2554\ (\text{FFW})$$
$$r = 0.9945;\ s_{yx} = 2.154.$$
$$\text{Total ash} = 0.94 + 0.03356\ (\text{FFW})$$
$$r = 0.9623;\ s_{yx} = 0.763.$$

The linear regressions account for 99.7, 98.9 and 92.6 of the variation for the total body water, protein and ash relationships, respectively.

Because of the different groups involved in the regressions, covariance analysis was necessary in order to determine whether all the lines of the individual subgroups were parallel; that is, if there were no significant differences in the slopes of the different subgroups. According to this analysis, a proof of the homogeneity of the variances is necessary before the comparison is made. Therefore, the Bartlett test for the homogeneity of variances was performed with the total weight of body water, protein, ash and fat-free body weight data. The results of the test gave chi-square values of 13.58, 19.49, 45.44 and 14.34, respectively, with 11 degrees of freedom. Only the chi-square test for the ash content was significant. On this basis, therefore, the analysis of covariance technique can be adequately applied only in the relationships water/fat-free weight and protein/fat-free weight. Because of the close association of total water and total protein to the fat-free weight, the correlation between water and protein was calculated. This correlation was also highly significant and the regression equation was:

$$\text{Total protein} = -1.70 + 0.3658\ (\text{total water})$$
$$r = 0.9905;\ s_{yx} = 2.847.$$

The analysis of covariance of the different regression lines in which the variances were homogeneous and also in the case of ash content is shown in table 3. The regression coefficients for the individual subgroups were not significantly different and the regression lines did not deviate significantly from the common regression. The adjusted means of the subgroups showed significant differences. Table 4 shows the average total weight body components for the different groups studied. They summarize the individual values used in the calculation of the different regressions given above.

DISCUSSION

If the level of protein in the diets, the age of the animals and the length of time they were fed the experimental diets were considered, it could be logically expected that the animals in the low protein diet group would show marked differences in body composition. The results presented in this paper, when expressed on a percentage basis, either in terms of fresh tissue or in fat-free weight, give no indication of marked differences in body components. The significant differences pointed out, although of statistical value, failed to give evidence of a profound effect produced by the level of the protein fed. During starvation, there is a marked reduction in the percentage of fat in the group previously fed the 5% casein diet, indicating a faster depletion of their fat reserves. The differences in the percentage of ash throughout the experimental

TABLE 3

Analysis of covariance with total body components [1]

	df	Fat-free weight			Water-protein
		Water	Protein	Ash	
Within	46	3.845	3.632	0.408	5.355
Regression coefficient	11	4.816	2.274	0.657	3.630
Common	57	4.033	3.370	0.456	5.022
Adjusted means	11	33.369*	11.022*	1.239*	24.082*

[1] Mean squares.
* Significant at $P < 0.01$.

TABLE 4

Effect of acute starvation and refeeding on total body components of rats previously fed different levels of dietary protein

	Starvation, days								Refeeding			
	0		2		4		6		5% casein		20% casein	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	Fat-free wet weight, g											
LP [1]	69.7	15.4	62.4	13.1	62.1	14.1	57.1	9.4	64.9	12.2	92.9	6.8
HP	232.8	22.8	225.5	27.1	205.4	22.4	208.7	23.6	216.6	27.7	230.8	22.9
	Total fat, g											
LP	12.7	4.8	9.5	4.2	6.8	1.0	3.6	1.3	8.4	2.5	13.4	2.5
HP	34.0	13.9	24.5	8.3	28.3	13.6	22.2	7.1	33.2	7.2	34.2	11.6
	Total water, g											
LP	48.5	10.9	42.6	9.0	42.0	9.6	39.3	6.3	46.5	8.4	68.7	5.1
HP	163.2	15.9	158.0	17.8	138.9	14.1	141.5	13.5	153.1	19.6	162.6	16.4
	Total protein, g											
LP	16.1	3.8	15.1	2.9	14.4	3.4	13.3	2.2	13.9	3.2	19.5	2.4
HP	59.2	6.2	54.4	7.9	51.3	7.3	53.3	7.1	53.9	8.0	54.7	4.8
	Total ash, g											
LP	3.4	0.3	3.0	0.6	3.2	0.6	2.8	0.4	3.2	0.6	3.8	0.3
HP	8.5	0.7	7.8	2.1	8.0	0.8	7.9	1.3	9.2	2.2	8.5	1.2

[1] LP indicates low protein; HP, high protein.

periods appear to be valid and consistent. The groups fed the 5% casein showed higher values than those fed the diet containing 20% casein.

The results presented here confirm the conclusion of Weil and Wallace (6) who have shown that animals under a variety of conditions of growth and suboptimal nutrient intake have a remarkably constant body composition per unit of body size. Widdowson and McCance (3) have shown that neither rats maintained with poor protein diets nor well-nourished rats subjected to starvation appear to have significant changes in their concentration of body protein on a fat-free basis. It can be added from the present work that animals with different levels of protein nutrition, especially malnourished animals during starvation, also showed a remarkable constancy of body composition, particularly on a fat-free basis.

The expression of body composition in terms of percentages or ratios has been criticized and the comparison of groups in terms of such averages may be misleading (12). In this respect, the comparison of the average percentages suggested some differences among the groups studied. However, the correlation coefficients showed a very close association between grams of fat-free weight and grams of total body water, protein or ash regardless of the dietary group or experimental condition. Also, the single linear regression lines account for more than 98% of the

variation for water and protein to fat-free weight, and more than 92% for ash to fat-free weight. The extreme closeness of fit suggested that the differences indicated by group comparisons might be "random phenomena or of questionable biological significance" (12).

It thus appears that the constancy of the composition of the lean body mass holds for animals in extreme levels of protein nutrition and also in cases of superimposed stress of acute starvation or refeeding after starvation. These relationships show that the body composition of the growing animals is size-dependent and that the dietary treatments and experimental conditions imposed had no effect on the relative distribution of body components, independent of its body size. The relationship of body composition and size has been proposed previously for ruminants by Reid et al. (13) and for the rat by Weil and Wallace (6). This conclusion, however, does not apply to animals after maturity, when growth has stopped and body fat becomes the only component dependent on diet.

The data presented here preclude the possibility that animals which consumed diets low in protein content and whose daily intake is lower would accumulate less body fat, assuming that the low food intake would not satisfy their caloric requirements. The percentage of body fat of the protein-malnourished animals was as high as that of the animals given the high protein diet. This observation has been indicated previously by Wallace.[5] It appears, therefore, that rats under dietary restriction during growth, adapt their metabolism to the diet, stopping their somatic growth. As a consequence, this results in small bodies with a harmonic distribution of body components which did not differ greatly from that in animals which had been maintained under optimal dietary conditions whose body size is three to four times greater.

Although body weight has been widely used as a measure of body size in nutrition, it has been severely criticized on the basis of its poor definition of body components. The information presented here, as well as the evidence given by others (6, 13), however, supports very strongly the use of body weight in growing animals as one of the best indicators of nutriture. Body weight, therefore, should be used with more assurance in the evaluation of the nutritional status in man and for the classification of malnutrition in children (14).

Although the results from animal experimentation should be extrapolated to humans only with caution, the possibility exists that the population groups in many of the developing areas which have subsisted under restricted diets, especially in quantity and quality of their dietary protein, could have a similar mechanism of adaptation to that discussed above. This would result in decreasing the rate of growth, giving small bodies but with a relatively similar composition to that of populations consuming adequate diets. It has been reported in this respect that the rural Guatemalan Indians (15) are shorter and lighter than the well-nourished urban population in Guatemala City. The weight-to-height ratio, however, is similar in both population groups when compared at the same heights, probably indicating a decrease in the rate of growth. The clinical impression is that rural Guatemalan Indians are well-endowed with muscle mass, although their skinfold thickness is small. These observations are also confirmed by different anthropometric measurements (15).

In the rat, protein nutrition or starvation of animals having different levels of protein nutrition, or refeeding after starvation, does not produce drastic changes in the composition of the lean body mass. This lends a good degree of confidence for the application of methods involving the assumption of the constancy of the lean body mass, to the study of body composition in population groups of different nutritional status or racial origin.

In addition, the close association of body size and body components in the growing rat also supports the use of body weight as a valuable index in the biological assay of protein quality. In this way not only the values derived from body weight per se can be calculated, but all other indexes involving body components as well.

[5] See footnote 3.

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Invitation for Nominations for 1967 American Institute of Nutrition Awards

Nominations are requested for the 1967 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. *Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1966,* to be considered for the 1967 awards.

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1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
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1951 – no award
1952 – H. E. Sauberlich
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1967 Conrad A. Elvehjem Award for Public Service in Nutrition

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

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Invitation for Nominations for 1967 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

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Send nominations to:

DR. S. L. HANSARD
Department of Animal Science
Louisiana State University
Baton Rouge, Louisiana 70803

The following persons have been elected previously as Fellows of the Society:

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Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

PAUL GYÖRGY, *Chairman*
GRACE GOLDSMITH
R. W. ENGEL

Send nominations to:

DR. PAUL GYÖRGY
University of Pennsylvania
Pennsylvania General Hospital
Philadelphia, Pennsylvania 19104

The following persons have been elected previously as Honorary Members of the Society:

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