Fatty Acid Composition of Testicular Tissue from EFA-deficient Swine [1]

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ABSTRACT A low fat casein-glucose monohydrate-type purified basal diet was used to study EFA deficiency in young uncastrated male swine. Gross dermal lesions, typical of EFA deficiency, were readily produced in pigs receiving the basal diet after 6 weeks on experiment. These dermal manifestations became increasingly severe as the experiment progressed, whereas pigs receiving the basal diet supplemented with 4% corn oil showed no evidence of skin lesions. Analyses of testicular tissue samples obtained from the pigs receiving the low fat basal diet showed a significant decrease in the dienoic and tetraenoic fatty acids with a corresponding increase in the trienoic fatty acids as compared with pigs receiving the diet containing corn oil. There was also a significant increase in the oleic acid and palmitoleic acid content of the EFA-deficient pigs. No differences were obtained in weight gains between pigs receiving the low fat diet and those fed the diet supplemented with corn oil over the 10-week experimental period.

A fat deficiency syndrome in swine, characterized by dermal lesions, was first described by Witz and Beeson (1), but no attempt was made to study involvement of specific fatty acids. More recently Hill et al. (2, 3) demonstrated variations in fatty acid composition of heart and liver tissue obtained from essential fatty acid-deficient swine, which was characterized by diminished dienoic and tetraenoic fatty acids and elevated trienoic fatty acid, but dermal lesions were not produced.

Since it is necessary to kill the experimental animal in obtaining tissue samples such as heart and liver for analytical procedures in studying the essential fatty acid (EFA) status of the pig, this necessarily allows only one analysis during a nutritional study. Obviously, it would be advantageous if some method were available whereby satisfactory tissue samples could be obtained for fatty acid analysis which would reflect EFA status and yet preserve the integrity of the experimental subject for further experimentation. Since the fatty acid composition of testicular tissue has been shown to be an indicator of the EFA status of the rat (4), it appears likely that testicular samples obtained periodically for tissue analysis from intact male pigs would offer a useful technique for the study of the EFA requirements in this species. The present study was therefore undertaken to develop a purified diet with which gross EFA deficiency symptoms could be produced in swine and to study the fatty acid composition of testicular and scrotal fat tissues in conjunction with the deficiency state.

EXPERIMENTAL

Eighteen uncastrated male pigs of Duroc and Landrace breeding were removed from their dams at an average age of 21 days. The pigs were assigned to 3 groups of 6 pigs each on the basis of breed and litter, with the outcome groups being allotted at random to treatment. Pens with wire mesh floors were used for rearing the pigs and the animals were housed in a pig nursery equipped with infrared heat lamps and ventilating fans. An orchiectomy was performed on the pigs in one group at the time of allotment to secure testicle and scrotal fat samples for fatty acid analyses to serve as a reference for the fatty acid status of the pigs at the start of the experiment. The other 2 groups were fed the purified diets shown in table 1. Diet A was formulated to provide a

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TABLE 1
Composition of diets

	Diet A	Diet B
	%	%
Casein	17.88	17.88
Glucose monohydrate	75.60	71.60
Powdered cellulose [1]	3.00	3.00
Corn oil	—	4.00
Defluorinated phosphate	2.10	2.10
Mineral mixture [2]	1.14	1.14
Vitamin mixture [3]	0.28	0.28

[1] Solka Floc, Brown Company, Chicago.
[2] Supplied the following quantities per kg of diet: (in grams) NaCl, 2.99; K_2HPO_4, 2.22; KCl, 3.83; $MgCO_3$, 1.52; $FeSO_4 \cdot 7H_2O$, 0.55; and (in milligrams) $MnSO_4 \cdot H_2O$, 135; $CoCl_2$, 26; $CuSO_4$, 5.50; $ZnCO_3$, 50; and KI, 3.0.
[3] Supplied the following quantities per kg of diet: vitamin A, 6600 IU; vitamin D_2, 1760 IU; α-tocopheryl acetate, 22 IU; and (in milligrams) menadione, 4.40; ascorbic acid, 55; thiamine·HCl, 5.5; riboflavin, 11; Ca pantothenate, 22; niacin, 66; choline chloride, 1100; *p*-aminobenzoic acid, 18; inositol, 198; pyridoxine·HCl, 5.50; streptomycin sulfate, 22; chlortetracycline, 22; and (in micrograms) biotin, 66; vitamin B_{12}, 44; and folic acid, 440. An antioxidant (ethoxyquin) was used at a level of 0.0125% in all diets.

low fat basal diet, and diet B was similar plus the addition of corn oil as a source of essential fatty acids. Analysis of the low fat basal diet (diet A) showed a total lipid level of 0.19% with a linoleic acid content of 0.02% of the diet. Diet B contained 4.06% total lipid and 2.19% linoleic acid. Feed and water were provided ad libitum.

After the pigs had received the experimental diets for a period of 5 weeks, an orchiectomy was performed on each pig in which the left testicle and a sample of scrotal fat were obtained and immediately frozen until analysis for constituent fatty acids could be carried out by gas liquid chromatography. When the pigs had received the experimental diets for 10 weeks, the remaining testicle and a scrotal fat sample were obtained from each pig for fatty acid analysis.

The lipid components were extracted using the method of Folch et al. (5) and the methyl esters were prepared as described by Knight et al. (6). Analyses of the fatty acid methyl esters were performed on a hydrogen flame ionization detector-type gas-liquid chromatographic apparatus using diethylene glycol succinate polymer as the stationary phase. Identification of the fatty acid esters was by the use of standards obtained from the Hormel Institute, Austin, Minnesota.[2] Quantitative results agreed with the stated composition data with a relative error of less than 2% for major components (> 10% of total mixture) and less than 6% for minor components (< 10% of total mixture).

[2] Standards used in identifying the fatty acid esters were Hormel model mixtures nos. 2 and 4 in which 8 components were identified directly.

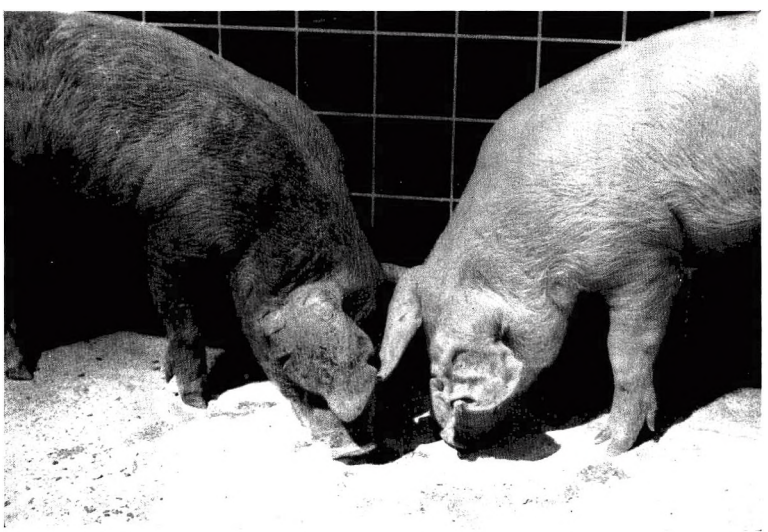

Fig. 1 Littermate pigs that were fed diets deficient in essential fatty acids (left) and adequate in fatty acids (right). Note skin lesion posterior to the ear and profuse accumulations of brown exudate over the dorsal surface of the EFA-deficient pig (left).

Statements of significance are based on results of analysis of variance of data collected.

RESULTS AND DISCUSSION

Dermal lesions, typical of EFA deficiency, began to appear among the pigs receiving the low fat basal diet (diet A) during the sixth week on experiment and increased in severity with time. These lesions were characterized at first by a scaly, dandruff-like desquamation of the epithelium across the dorsal surface. The hair was dull and dry. A brownish, gummy exudate appeared first about the ears, axillary spaces and under the flanks as shown in figure 1. The skin on the tails of the pigs was dry and scaly with some necrotic areas being observed on some pigs during the terminal stages of the experiment. Skin eruptions were present about the ears, axillary spaces and flanks in the severest cases. Toward the end of the experiment, the pigs were profusely covered over the dorsal surface with the gummy exudate. None of these symptoms were present among the pigs receiving the diet supplemented with corn oil (diet B).

No difference in gains was obtained between the pigs fed the 2 diets (table 2). Efficiency of feed utilization was slightly greater for the pigs receiving the diet supplemented with corn oil, which is explained by the slightly higher caloric density of this diet.

Fatty acid composition data for the testes tissue are presented in table 3. Data for the testes removed from the one group of pigs at the start of the experiment, when they were first removed from their dam, are included for reference. Highly significant differences were found in the fatty acid composition of the testicular tissue from pigs fed the low fat basal diet as compared with those receiving the diet supplemented with corn oil. A highly significant decrease in the dienoic and tetraenoic fatty acids occurred in the pigs receiving the low fat basal diet with a corresponding increase in the content of trienoic fatty acids. These changes are similar to the biochemical lesions which have been reported to be characteristic of EFA-deficiency in rats (7), chicks (8), dogs (9) and swine (3). It is interesting to note that these changes in fatty acid composition were clearly evident in the testes tissue obtained after only 5 weeks of feeding the experimental diet, which was prior

TABLE 2

Gain and feed utilization data

	Basal diet	Basal + CO
No. of pigs	6	6
Initial wt, kg	5.63	5.68
Daily gain, kg	0.51	0.50
Feed/gain	2.13	1.94

TABLE 3

Fatty acid composition of testicular tissues expressed as percentage by weight of the total fatty acids [1]

Fatty acid	Reference pigs [2]	Basal diet		Basal + CO	
		5 weeks	10 weeks	5 weeks	10 weeks
14:0	1.72	0.75	0.68	0.95	0.90
16:0	24.57	23.42	22.30	24.59	25.44
16:1	2.94	4.76	4.73	2.77[aa]	2.47[bb]
18:0	12.38	15.92	14.68	16.02	16.10
18:1	12.45	28.08	31.01	15.15[aa]	15.51[bb]
18:2	13.80	2.68	2.34	10.13[aa]	9.80[bb]
18:3	trace	1.04	0.83	0.90	0.33[b]
20:0	1.50	0.40	0.19	1.30[aa]	1.03[bb]
20:3	2.61	10.90	14.68	3.23[aa]	3.35[bb]
20:4	13.80	6.80	2.35	17.17[aa]	15.20[bb]
X[3]	14.23	5.25	6.21	7.79	9.87

[1] Data from 6 pigs included in each mean.
[2] Analysis of testes obtained from 21-day-old pigs at beginning of experiment.
[3] Unidentified fatty acid esters having GLC retention times greater than 20:4.
[aa] Significant difference between treatments at 5 weeks ($P < 0.01$).
[b] Significant difference between treatments at 10 weeks ($P < 0.05$); [bb] ($P < 0.01$).

TABLE 4

Fatty acid composition of scrotal fat expressed as percentage by weight of the total fatty acid

Fatty acid	Reference pigs [1]	Basal diet		Basal + CO	
		5 weeks	10 weeks	5 weeks	10 weeks
14:0	2.42	1.49	1.56	1.56	1.56
16:0	23.12	25.71	26.58	22.99	25.36
16:1	6.79	6.47	5.75	5.03	4.32
18:0	5.94	10.69	11.64	10.02	12.15
18:1	43.90	50.40	52.78	44.79[aa]	44.97[aa]
18:2	11.26	3.67	1.20	13.00[aa]	10.61[aa]
18:3	1.30	1.47	0.45	1.37	0.85

[1] Analysis of scrotal fat obtained from 21-day-old pigs at beginning of experiment.
[aa] Significant difference between treatments ($P < 0.01$).

to the development of observable dermal lesions. A highly significant increase in oleic acid (18:1) and palmitoleic acid (16:1) also was noted in the testicular tissue from pigs receiving the EFA-deficient basal diet. The fatty acid composition of testes tissue from the 21-day-old pigs and those receiving the diet supplemented with corn oil was quite similar.

Data for the fatty acid composition of the scrotal fat are shown in table 4. The only significant differences in the fatty acid content of depot fat from pigs fed the 2 diets was a highly significant decrease in linoleic acid (18:2) and a highly significant increase in oleic acid (18:1) in the fat from pigs fed the low fat basal diet.

Dermal lesions observed among pigs on this experiment are similar in many respects to those reported originally by Witz and Beeson (1). The biochemical lesions are in agreement with those reported by Hill et al. (2, 3) for swine. However, the failure to obtain a difference in growth rate between pigs fed the low fat diet and those receiving the diet containing the corn oil supplement in this study is somewhat unexpected in view of reports indicating marked effects on gains of EFA-deficient pigs. For instance, Hill et al. (3) reported significant increases in weight gains of pigs fed diets adequate in EFA content as compared with pigs fed a deficient diet. Witz and Beeson (1) observed differences in gains in one experiment, but not in the other. The gains of the pigs in the experiment reported here are excellent for pigs during this stage of growth, which is ample evidence that the purified basal diet employed was nutritionally adequate in all respects except for fat content. It therefore appears that growth rate is not a reliable criterion in ascertaining EFA-deficiency in the pig. Criteria such as the biochemical lesions characterized by marked alteration in tissue fatty acid composition and correlated with gross dermal manifestations are apparently much more reliable in determining the EFA status of swine. The technique of using uncastrated male pigs as experimental subjects, where testes and scrotal fat samples can be readily obtained for analytical procedures at different stages of an experiment, provides a convenient method for the objective evaluation of the EFA status of swine.

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Amino Acid Requirements of Grain Beetles [1,2]

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ABSTRACT Development of a diet purified with respect to amino acids has led to the determination of amino acid requirements of several grain beetles. The confused flour beetle, *Tribolium confusum* (Duval), required (in per cent of diet) arginine, 0.5; histidine, 0.2; isoleucine, 0.5; leucine, 0.8; lysine, 0.56; methionine, 0.3 (0.15 with excess cystine); phenylalanine, 0.7 (0.4 with excess tyrosine); threonine, 0.4; tryptophan, 0.1; and valine 0.6. The red flour beetle, *Tribolium castaneum* (Herbst), required (in per cent) arginine, 0.4; histidine, 0.2; isoleucine, 0.3; and leucine, 0.7. The saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.), required (in per cent) arginine, 0.3; histidine, 0.2; isoleucine, 0.3; and leucine 0.6. Other amino acid requirements for the latter 2 insects were not determined. Requirements of these grain insects are compared with those of other organisms. The confused flour beetle was tested as an assay organism for amino acid content of protein but showed little promise.

Due mainly to the difficulties involved in preparing diets adequately purified for the study of quantitative nutritional requirements, most nutritional investigations of grain insects, and of insects in general, have been of a qualitative nature (1–4).

In the present study, emphasis was placed on the quantitative amino acid requirements of the confused flour beetle, *Tribolium confusum* (Duval), while the requirements of the red flour beetle, *Tribolium castaneum* (Herbst), and the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.), for 4 amino acids were also determined. The confused flour beetle requires the same 10 amino acids required by many vertebrates (5), and the relative order of protein quality for larvae of this insect is similar to that for the rat (3). Most other grain insects also require these 10 amino acids (2).

The purposes of this study were to obtain basic information on the nutrition of insects and to acquire enough information on the confused flour beetle to determine whether this insect might be used as an assay organism for the amino acid content of feedstuffs.

EXPERIMENTAL

The composition of the control diet which was used in all experiments is shown in table 1. This diet is nutritionally adequate, since it supports rapid growth of all insects studied for at least 3 generations. In each test, the amino acid being investigated was fed in a series of diets at different levels, the levels of the other dietary components being kept the same as those of the control diet. All insect colonies were reared in a constant temperature room at 28° with a stock diet containing 95% unbleached wheat flour and 5% dried yeast. Eggs were sifted from this stock diet 24 hours before the start of each experiment. Three groups of ten each, newly emerged larvae (average age 12 hours) per level of nutrient tested were placed in small vials containing the experimental diets. Such replicates seldom varied more than 8% from the average of the 3 values and if so were discarded. The least difference between averages required for significance at the 1% level was 0.123 mg/larvae. The flour beetle larvae were incubated at 28° and 75 ±5% relative humidity for 18 days, whereas the larvae of the saw-toothed grain beetle were incubated for only 14 days. At the end of the 14- or 18-day period, the surviving larvae were separated from the diet, counted and weighed. Usually all 10 larvae survived. A comparison of the final weights with the

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TABLE 1

Composition of control diet used in all experiments

	%
Cornstarch	72.5
Amino acids (all L-) [1]	20.0
Minerals [2]	3.5
Corn oil	3.0
Vitamin mixture [3]	0.63
Cholesterol	0.37

[1] Composition of amino acid mixture: (as per cent of diet) arginine, 0.9; histidine, 0.6; isoleucine, 1.2; leucine, 1.4; lysine·HCl, 1.3; methionine, 0.6; phenylalanine, 0.8; threonine, 0.9; tryptophan, 0.2; valine, 1.1; glutamic acid, 4.5; glycine, 4.5; and cystine, 2.0. Levels of the essential amino acids were based on the levels present in good quality proteins.

[2] Ash of whole wheat.

[3] Vitamins used were: (in micrograms/gram of diet) choline chloride, 4000; nicotinic acid, 100; Ca D-pantothenate, 40; riboflavin, 18; pyridoxine·HCl, 16; thiamine·HCl, 12; folic acid, 5; biotin, 0.6; carnitine, 10; inositol, 2000; *p*-aminobenzoic acid, 500; L-ascorbic acid, 10; menadione, 1; and cobalamin, 0.5. The last 5 vitamins are not among the known requirements of *T. confusum*. Carnitine is required for pupal emergence only.

experimental diets and final weights with the control diet (table 1) was used to evaluate the experimental diets.

Figure 1 is a plot of the data from the isoleucine and valine experiments. These 2 amino acids represent extremes encountered with respect to slopes of the curves. The other 10 curves, including experiments on the sparing actions of cystine and tyrosine, ranged between these two. The point of maximal inflection in the upper portion of each curve was assumed to show the minimal requirement for the amino acid concerned. Larval growth with the control diet was also considered in each experiment as a check on the constancy of experimental conditions. The noticeably lower growth with valine was below what is considered normal and was due to excessive vibration of the flasks from a nearby shaking machine.

Two other amino acids, as well as the control diet, run concurrently, gave similarly low growth. When the incubator was removed to a quiescent spot for later assays, growth with the control and experimental diets returned to the higher figures.

It was not possible to weigh individual larvae because of time limitations and because the larvae dehydrated on exposure to air; it was not possible, therefore, to run detailed statistical analyses. In a few cases where this was tried there was only moderate scatter. There were occasional very small individuals (runts), but the incidence was low. In over a thousand observations the numbers ranged from 1.4% with good diets to 6.3% with poor diets.

Statistical analysis on responses with the control diet, which was run with each group of assays, indicated an undetermined source of variation occurring over a period of months or years. Therefore, weights obtained in experiments run at different times may not reliably be compared with each other, but must be considered with respect to a standard such as the control diet. In the present work each experiment was self-contained so that such a comparison was largely unnecessary.

RESULTS AND DISCUSSION

The results of the 12 experiments on the confused flour beetle and the 4 experiments each on the red flour beetle and the saw-toothed grain beetle are summarized in table 2. For comparison this table also contains information on the requirements of other organisms. The honey bee is the only other insect that has been completely studied in terms of amino acid requirements.

In general, the requirements for the insects are higher for the larger insects (honey bee and confused flour beetle) and

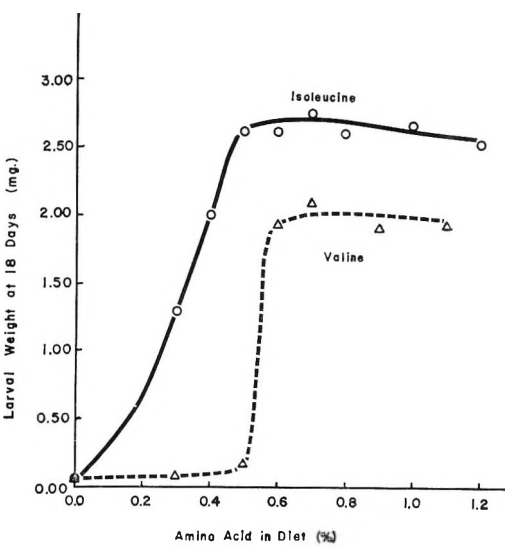

Fig. 1 Weight of larvae of the confused flour beetle at 18 days fed at graded levels of isoleucine and valine.

TABLE 2

Amino acid requirements of grain beetles with comparison of requirements of certain other species [1]

Amino acid [2]	Confused flour beetle larva	Red flour beetle larva	Saw-toothed grain beetle larva	Honey bee (6)	Young rat (7)	Adult rat (7)	Young chicken (8)	Young human (9) [3]	Adult human (9) [3]
Arginine	0.5	0.4	0.3 [4]	0.6	0.2	—	1.2	—	—
Histidine	0.2	0.2	0.2	0.3	0.3	0.07	0.3	?	—
Isoleucine	0.5	0.3	0.3	1.0	0.5	0.43	0.6	0.6	0.6
Leucine	0.8	0.7	0.6	0.9	0.8	0.25	1.4	2.8	0.9
Lysine	0.56	?	?	0.6	0.9	0.14	1.0	1.1	0.6
Methionine no cystine	0.3	?	?	?	0.6	0.23	0.8	?	?
excess cystine [5]	0.15	?	0.14(11)	0.3	0.3	0.11	0.45	0.6	0.2
Phenylalanine no tyrosine	0.7	?	?	?	0.9	?	0.7	1.9	?
excess tyrosine	0.4	?	?	0.5	0.6	0.19	0.14	1.1	0.9
Threonine	0.4	?	?	0.6	0.5	0.17	0.6	0.6	0.4
Tryptophan	0.1	?	?	0.2	0.15	0.07	0.2	0.2	0.2
Valine	0.6	?	?	0.8	0.7	0.31	0.8	1.8	0.7
Glycine	—	—	—	—	—	—	1.0	—	—

[1] All requirements expressed as percentage of diet.
[2] All grain insect requirements shown are for L-amino acids.
[3] Allison, J. B. 1960 The ideal aminogram. Fifth International Congress of Nutrition, p. 6.
[4] Davis (8) reports a value of 0.33%.
[5] Excess cystine means an amount which we determined to be somewhat above the level giving a response with a minimum of methionine but not giving any indication of toxicity.

lower for the smaller insects (red flour beetle and saw-toothed grain beetle). As an hypothesis, it is suggested that these differences in the requirements of three such similar insects may be associated with the ad libitum feeding techniques which must necessarily be used with such small organisms. The smaller insects, having higher metabolic rates because of the greater surface area per unit volume and thus higher rates of feeding, might be expected to eat more food per unit of body weight, and thus would show lower requirements in such feeding experiments. Rate of growth should also be a factor. More data on a variety of other insects would be needed to test this theory.

The principal amino acid which is required in markedly different amounts by insects, as compared with higher organisms, is methionine. The higher requirements of mammals and the chicken may be necessary for supplying sulfur amino acids for the production of hair and feathers. Arginine shows considerable variation among the various species.

It was found that the confused flour beetle's growth was slightly improved when glycine was used as the supplier of extra nitrogen in a diet containing minimal levels of the essential amino acids. This may be due to the method of nitrogen excretion. Like the chicken, which requires glycine, insects excrete nitrogen largely in the form of uric acid.

When the essential amino acids were fed to the confused flour beetle at their determined minimal required levels in a diet containing adequate nonessential amino acids, considerably lower growth was observed than that with the control diet. Additional amounts of certain amino acids, selected on the basis of some uncertainty as to the exact point of inflection of the growth response curve, improved growth somewhat, as shown in table 3, but

TABLE 3

Growth of confused flour beetle larvae fed diets containing minimal levels of the essential amino acids

	Variable amino acids [1,2]					Larval growth [3]
	His	Ileu	Leu	Phe	Try	
	%	%	%	%	%	*mg*
Minimal levels	0.2	0.6	0.8	0.7	0.10	1.76
	0.3	0.6	0.8	0.7	0.10	1.75
	0.4	0.6	0.8	0.7	0.10	1.90
	0.4	0.7	0.8	0.7	0.10	1.97
	0.4	0.6	0.8	0.7	*0.12*	2.15
	0.4	0.6	0.8	*0.8*	0.10	2.19
	0.4	0.6	*0.9*	0.7	0.10	2.17
	0.4	0.6	*0.9*	*0.8*	*0.12*	2.25
Control diet	0.6	1.2	1.4	0.8	0.20	2.50

[1] Other dietary ingredients were the same as those in the control diet except for increases in glycine and glutamic acid to maintain a total of 20% amino acids.
[2] Levels increased where italicized.
[3] Average of 30 larvae at the end of 18 days' incubation.

growth still failed to reach that obtained with the control diet. It appears that, for optimal growth, several of the amino acids should be present at much more than the minimal requirement level, or that the requirements for various amino acids is lower in the presence of an excess of the other essential amino acids. Thus the requirement for histidine was raised from 0.2 to 0.4% when the low levels of the other acids were used.

An assay procedure for amino acids, using confused flour beetle larvae, was tested on samples of gelatin, casein hydrolysate, and sesame meal which had been analyzed by automatic column chromatographic techniques. Leucine and isoleucine were chosen for assay and the proteins were added to the control diet to furnish graded levels of the amino acids, the amino acid itself being omitted. The glycine and glutamic acid content of the control diet was reduced to 1% each to balance somewhat the added nitrogen from protein.

Results of the assays were unexpected. Gelatin gave very poor growth at all levels and hence was considered as toxic to the flour beetle. Casein hydrolysate gave opposite results; even the low levels, furnishing leucine and isoleucine much below the minimal requirement, gave growth of 2.5 to 2.9 mg in 18 days.

Sesame meal was the only test material which behaved in what might be called a normal manner. When it furnished 0.3% isoleucine, growth was the same as that when 0.3% isoleucine was supplied as the free acid. However, at 0.5% isoleucine, the requirement level, growth was much superior with the sesame meal, and at 0.6% isoleucine from sesame growth was 3.34 mg, a value never reached with the control diet. It appears from these 3 assays that there may be complicated interrelations in the amino acid requirements of this insect. The casein and sesame meal may have also contained non-amino acid growth factors of the type suggested by Horn and Warren (12) in their work with microorganisms.

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Mineral Requirements of the Confused Flour Beetle, *Tribolium confusum* (Duval) [1,2]

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ABSTRACT The mineral requirements of the flour bettle, *Tribolium confusum* (Duval), were determined through use of a basal diet which contained 8 mineral salts as the mineral supplement. Larvae of the flour beetle require: (in parts per million in the diet) potassium, 2000; magnesium, 200; iron, 10; zinc, 5; and manganese, 1.5. Requirements for calcium, sodium, and copper are less than 2.1, 100, and 0.12 ppm, respectively, the amounts present in the unsupplemented diet. Toxicities of the salts used in these tests were also determined. Levels of the salts which were tolerated varied between 0.15 and 5.0% of the diet, depending on the individual salt. The mineral requirements of the confused flour beetle are compared with those of other organisms.

In research on insect nutrition, there have been few significant investigations of mineral requirements (1–3). In attempts made to study this area, the experimental diets could not, for the most part, be so purified as to allow determination of absolute mineral requirements and, as a result, only one or two of the cations were studied (4–6).[3]

In some cases, mineral mixtures used in basal insect diets were similar to those used in vertebrate nutrition (7, 8),[4] but we feel that mixtures containing minerals at levels similar to those present in the natural food products of the insects represent the best diets for such studies. Such mixtures, usually containing more potassium and magnesium (present in high levels in plants) and less calcium (trace element for most insects), have been used, often with fairly good results, in studies of mineral requirements of certain insects (3, 9–11). The present study involved use of such a diet in an investigation of the mineral requirements of the confused flour beetle, *Tribolium confusum* (Duval).

EXPERIMENTAL

During previous experiments on the amino acid requirements of grain beetles (12) a purified diet, called the control diet, was developed which gave excellent growth of larvae. This diet could not be used to determine mineral requirements, however, because inorganic elements were supplied by an empirical mixture, the ash of whole wheat. It was necessary to develop a mixture of known pure salts in order to carry out the determination of mineral requirements.

The composition of the mineral mixture finally arrived at (table 1) was based on flame photometric and spectrographic analyses on various adequate diets for beetles, as well as on the beetles themselves in various stages of metamorphosis. The summary of dietary requirements of insects by Fraenkel [5] was also considered, as were the needs of various life processes, such as for co-factors for enzymes. Based on these considerations, 6 different mixtures were prepared and tested at the 2.0 and 3.5% levels, all containing the 6 salts suggested by Fraenkel for *Tenebrio molitor* (10) and some containing manganese and copper in addition.[6] The mixture which gave the best results (table 1) contained

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[6] See footnote 5.

TABLE 1

Composition of mineral mixture used in mineral control diet, and amounts of cations supplied

Salt [1]	Salt in mixture	Salt in diet	Cation in diet	
	%	%	%	*ppm*
KH_2PO_4	67.75	1.36	0.3930	3930
$MgSO_4$	16.0	0.32	0.0719	719
$Ca(H_2PO_4)_2 \cdot H_2O$	8.0	0.16	0.0257	257
NaCl	5.0	0.10	0.0493	493
$FeSO_4 \cdot 7H_2O$	2.0	0.04	0.0082	82.2
$ZnCl_2$	0.5	0.01	0.0049	49.2
$MnSO_4 \cdot H_2O$	0.5	0.01	0.0034	33.7
$CuSO_4 \cdot 5H_2O$	0.25	0.005	0.0013	12.8
Totals	100.0	2.00	0.5577	5576.9

[1] All salts were reagent grade.

considerably more magnesium and less sodium and zinc than did Fraenkel's mixture, and also contained manganese and copper. Since 3 successive generations of beetles reared with this diet have each shown a good rate of growth, this diet should be completely adequate for the growth and reproduction of flour beetles.

This final diet, called the mineral control diet, was much the same as the control diet used in previous studies (12) and had the following percentage composition: cornstarch, 74.0; amino acids (all L-), 20.0; reagent grade mineral salts, 2.0; corn oil, 3.0; complete vitamin mixture, 0.63; and cholesterol, 0.37. Care was taken to ensure a high degree of fineness and complete mixing. Changes from the control diet were: (in per cent of diet) phenylalanine reduced from 0.8 to 0.5, tyrosine (not previously used) 1.0, glutamic acid reduced from 4.5 to 3.0, glycine increased from 4.5 to 5.3, pure minerals 2.0 in place of ash of wheat 3.5, and cornstarch to make 100. Average larval weight with the mineral control diet was usually between 2.5 and 2.7 mg at 18 days and appeared to be very slightly better than that with the previously used control diet which averaged close to 2.5 mg.

Growth studies were carried out for the usual 18 days as described in our previous paper (12) with 3 groups of 10 newly emerged larvae being fed each level of the mineral being tested. Growth curves were similar to those obtained with amino acids except that, in some cases, diets could not be made sufficiently low in certain elements to get reduced growth and in all cases levels of the salts high enough to be toxic were fed. Curves illustrating typical responses are shown in figure 1 and the data for these curves and for the other elements tested are given in table 2.

The anions in the experimental diets were maintained at a uniform level, using calcium or potassium salts of the respective anions, since these cations are least toxic. Requirement and toxicity levels were determined as in the amino acid studies by inspection of the plots of dosage-growth response curves.

RESULTS AND DISCUSSION

A summary of the experiments on the requirements of the confused flour beetle

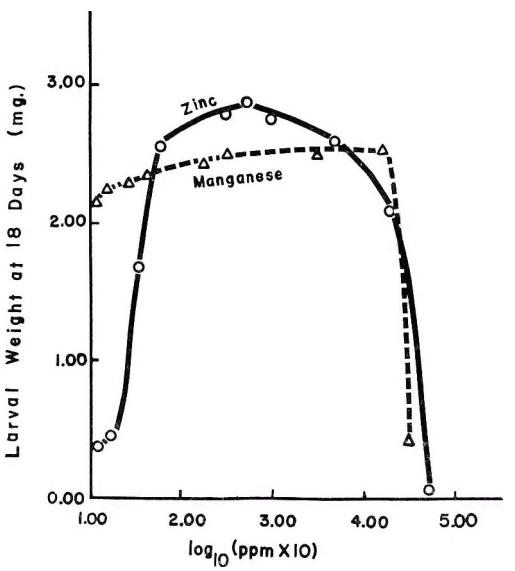

Fig. 1 Weight of larvae of the confused flour beetle fed at various levels of manganese and zinc. Levels are plotted logarithmically to condense the scale.

TABLE 2

Growth of flour beetle larvae fed diet at graded levels of minerals [1]

Salt added	Element in diet [2]	Wt of larvae [3]	Salt added	Element in diet [2]	Wt of larvae [3]
%	*ppm*	*mg*	%	*ppm*	*mg*
	Potassium			Magnesium	
(as KH_2PO_4)			(as $MgSO_4$)		
0.00	28.3	0.04	0.00	73	0.05
0.01	57.0	0.07	0.01	93	0.25
0.10	315	0.65	0.10	275	2.55
0.30	889	1.27	0.20	477	2.53
0.50	1470	1.06	0.25	578	2.71
0.75	*2180*	2.54	0.32	719	2.61
1.00	2900	2.55	1.00	2090	2.55
1.36	3930	2.51	2.00	4110	2.23
2.00	5770	2.64	5.00	10200	0.80
5.00	14400	1.66	10.00	20300	0.68
10.00	28800	1.05			
	Iron			Zinc	
(as $FeSO \cdot 7H_2O$)			(as $ZnCl_2$)		
0.0	1.8	0.71	0.0	1.2	0.37
0.0001	2.0	0.80	0.0001	1.7	0.45
0.001	3.8	1.10	0.0005	3.6	1.76
0.005	*11.9*	2.42	0.001	6.0	2.48
0.01	21.9	2.39	0.005	25.2	2.77
0.04	82.2	2.54	0.01	49.2	2.85
0.10	203	2.72	0.02	97.2	2.78
0.50	1007	1.65	0.10	481	2.59
1.00	2010	1.24	*1.00*	4810	0.70
	Manganese			Copper	
(as $MnSO_4 \cdot H_2O$)			(as $CuSO_4 \cdot 5H_2O$)		
0.0	1.2	2.12	0.0	0.12	2.56
0.0001	*1.5*	2.26	0.0001	0.37	2.56
0.0005	2.8	2.28	0.0005	1.39	2.54
0.001	4.5	2.33	0.001	2.66	2.51
0.005	17.5	2.38	0.002	5.20	2.74
0.01	33.7	2.44	0.005	12.8	2.16
0.10	326	2.42	0.02	50.9	2.78
0.50	1625	2.48	0.10	254	2.64
1.00	3250	0.40	0.20	508	0.88
			0.50	1270	0.04
			1.00	2540	0.04
	Calcium			Sodium	
(as $Ca(H_2PO_4)_2 \cdot H_2O$)			(as NaCl)		
0.0	2.1	2.42	0.0	*100*	2.18
0.0001	2.3	2.36	0.0001	100.4	2.54
0.0005	2.9	2.26	0.0005	102	2.67
0.002	5.3	2.44	0.002	108	2.32
0.01	18	2.51	0.01	139	2.42
0.16	257	2.64	0.10	493	2.61
1.50	2390	2.12	*1.00*	4030	0.17
4.00	6380	1.14	3.00	11800	(died)
10.00	15900	0.15	10.00	39400	(died)

[1] Values italicized are toxic levels of the salt fed and requirement for the cation.
[2] All weights are the average of surviving larvae from 3 test vials after 18 days of incubation.
[3] Amount present in unsupplemented diet.

TABLE 3

Mineral requirements of the confused flour beetle for cations, and toxic ranges of the mineral salts used

Cation	Cation in unsupplemented diet [2]	Minimal requirement	Estimated toxic levels of salts [1]	
	ppm	*ppm*		%
K	28.3	2000	KH_2PO_4	3.0–5.0
Mg	73	275	$MgSO_4$	2.0
Ca	2.1	2.1	$Ca(H_2PO_4)_2 \cdot H_2O$	1.5
Na	100	100	NaCl	0.2–0.6
Fe	1.8	10	$FeSO_4 \cdot 7H_2O$	0.3–0.5
Zn	1.2	5	$ZnCl_2$	0.2
Mn	1.2	1.5	$MnSO_4 \cdot H_2O$	0.6–0.9
Cu	0.12	0.12	$CuSO_4 \cdot 5H_2O$	0.15

[1] Changes in dietary levels of other minerals may change the toxicity range of some of these salts.
[2] Calculated from flame photometric and spectrographic determinations of the respective elements.

for 8 metal cations and on the toxicities of the respective salts (table 3) shows definite requirements for potassium, magnesium, iron, and zinc. A requirement for manganese is indicated in the table but not absolutely proved, since larval growth (2.12 mg) with the lowest level of manganese tested was not low enough to allow complete evaluation of the requirement. Figure 1 is a semilogarithmic plot of the data on the manganese experiment; included also is the plot of the experiment on the zinc requirement, which is more nearly ideal. Also, requirements for calcium, sodium, and copper could not be determined accurately because the amount of each present in the unsupplemented diet was sufficiently large to preclude benefit from further addition. These requirements, therefore, must be stated as not more than the given level.

Definite toxicity ranges for each of the salts fed were observed. The levels at which the salts became toxic varied considerably and showed little relationship to the requirements for the cations involved. In general, the toxicity appears much greater for the insect than is the case for higher animals. For example, it is common practice to use 1% sodium chloride in rations for rats and chickens. These observations are similar to those of Majunder and Bano (14), who reported pronounced toxicity of common salts, especially tricalcium phosphate, for several grain beetles. Our data for monocalcium phosphate agree well with theirs.

After the above mineral requirements for the flour beetle had been determined, a mixture of minerals containing the minimal required levels of each cation was prepared and tested as the mineral supplement in a purified diet. As in the amino acid studies with this insect when levels of these compounds were all reduced to minimal requirements (12), larval growth (1.77 mg) was poorer than with the control diet (2.51 mg). A higher level of magnesium (447 ppm) increased growth to 2.06 mg at the end of 18 days. Only 275 ppm of magnesium are required in a diet containing 2.0% minerals; thus more is required in the minimal diet which contains only 0.86% minerals. Apparently, minerals other than magnesium are also required in greater amounts in this minimal diet though these were not tested. The results suggest that the requirement for a given mineral may be lowered in the presence of an excess of certain others. Phosphorus was supplied at more than 2000 ppm by the potassium phosphate plus that present in the starch. The minimal level of magnesium sulfate supplied 270 ppm of sulfur, in addition to that supplied by 0.6% methionine and 2.0% of cystine. Lysine·HCl, at 1.3% of the diet, would furnish 2600 ppm of chloride. It appears, therefore, that common anions should not be lacking.

In table 4, the mineral requirements of the confused flour beetle are compared with those reported elsewhere for this insect and also with those reported for other organisms. The previously reported calcium requirement of the flour beetle by Huot et al. (13) is not in agreement with that reported here. This may be due to the

TABLE 4

Comparison of the mineral requirements of the confused flour beetle with those of other organisms

Element	Larva		Mealworm larva (10)	Young chicken (16)	Young rat (17)	Young human (18)
	Reported here	Reported elsewhere (13, 15)				
	ppm	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
K	2000	2700	2000	2000	1800	4000
Mg	275	200	400	500	400	300
Ca	< 2.1	140	80	10000	6000	1200
Na	< 100	< 1200		1500	500	1600
Fe	10			20	25	12
Zn	5		6	45	12	2
Mn	1.5			50	50	—
Cu	< 0.12			2	5	2.5
P		1000		6000	5000	1200
Cl				2200	500	2400
I				1	0.15	0.02
Se					0.04	

less adequate diet used by these investigators, although the difference appears to be too great to be explained in this way. With their diet, pupation of the larvae occurred at about 35 days, as compared with the pupation at between 19 and 23 days observed with the purified diets shown here. The calcium requirement could be altered under such circumstances.

The most striking feature of table 4 is that, compared with vertebrates, insects require so little calcium. This is reasonable, however, since insects, unlike vertebrates, do not have internal skeletons which contain calcium. Insects probably require calcium only for nerve-muscle integrity and possibly membrane permeability.

The flour beetle also requires less sodium and copper than vertebrates. The decreased sodium requirement has probably developed genetically, since, as these insects evolved, they fed on plants which contain lower amounts of sodium than animal products. The flour beetle's lower requirement for copper may be due to differences in the way insects and vertebrates manufacture the oxygen-carrying components of their blood.

The total amount of minerals which the flour beetle requires in its diet is considerably less than the level required by vertebrates. The necessity for water conservation in the flour beetle may account for this; vertebrates normally ingest and eliminate much greater amounts of water and thus eliminate greater amounts of minerals. The same reasoning probably explains the higher toxicity of common salts.

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Digestibility of Unheated Soybean Meal for Laying Hens [1]

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ABSTRACT The digestibility of protein and energy in diets containing heated and unheated soybean meal was determined with colostomized laying hens. The data obtained showed that protein in diets containing unheated soybean meal was only 54% digested compared with 85% for heated meal. The difference in protein digestibility was sufficient to account for the difference in energy digestibility. The metabolizable energy value of diets with heated or unheated soybean meal was determined after 12, 36, 67, and 97 days of feeding. There was no evidence of a change in the metabolizable energy value of the diets with time. Adult chickens did not respond differently from young chicks to diets containing unheated soybean meal. These experiments also showed that hens did not adapt, following a prolonged feeding period, so that they were better able to digest unheated soybean meal.

The utilization of unheated soybean meal by laying hens or by chickens over 6 to 8 weeks of age has been studied by several investigators with conflicting conclusions reported.

Fisher and Johnson (1) observed that diets containing unheated soybean meal supported egg production as well as diets containing heated soybean meal, although the minimal protein level necessary for normal egg production was higher when unheated meal rather than heated meal was fed. In studies by Saxena et al. (2) egg production, nitrogen retention and pancreas weight of hens receiving diets containing unheated soybean meal were the same as for hens receiving a similar diet containing heated meal. They also reported that chicks were less affected by unheated meal as they became older. They postulated that young chicks lack an enzyme that digests an "active" fraction of the meal but that hens possess this enzyme.

Similar results were reported by Bornstein and Lipstein (3). When response of chicks was measured as "percentage gain," chicks 11 to 12 weeks of age gained at the same rate whether they were fed diets containing heated or unheated soybean meal beginning at 8 weeks of age.

These reports appear to be supported by the observations by Alumot and Nitsan (4, 5) that although intestinal proteolysis was markedly depressed in young chicks fed unheated soybean meal, proteolysis gradually returned to normal levels during the fourth to sixth week of feeding. Proteolysis was measured in vitro from samples of intestinal contents. When unheated soybean meal was fed to older chickens, recovery of proteolysis was more rapid. When 6-week-old chicks were fed a diet containing unheated soybean meal, normal levels of proteolytic activity were restored between 2 and 4 days after starting to feed the diet.

Recent studies by Lepkovsky et al. (6) suggest that the level of proteolytic activity measured in the contents of the intestinal tract may not be a true reflection of the actual protein digestion taking place. These authors observed a higher percentage of nitrogen in the contents of the lower small intestine and feces of chicks given unheated meal compared with chicks receiving heated meal even though protease activity of the intestinal contents measured in vitro was the same for chicks receiving either diet. Overall nitrogen digestibility was markedly lower in ileostomized chickens fed unheated meal compared with those fed heated soybean meal.

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The metabolizable energy value of unheated soybean meal or soybeans was found to be the same for hens and chicks in studies by Hill and Renner (7, 8). Heat treatment of these soybean products markedly improved their metabolizable energy value. Egg production of hens receiving unheated meal was also depressed compared with that of hens receiving properly heated meal. Rogler and Carrick (9) also reported that hens fed diets containing unheated soybeans produced fewer eggs, had an enlarged pancreas and used feed less efficiently compared with hens receiving properly heat-treated soybeans. Egg production remained low for the whole 6-month period of feeding the unheated soybeans with no indication that hens eventually adapted to the soybeans.

The studies reported in this paper were designed to (a) measure apparent digestibility of nitrogen from unheated and heated soybean meal by hens prepared with a colostomy to permit separation of urine and feces, (b) compare digestible energy value of unheated or heated soybean meal to permit calculation of the utilization of non-nitrogenous components of the diet, and (c) determine whether metabolizable energy value of a diet containing unheated soybean meal changed over a prolonged feeding period.

EXPERIMENTAL

White Leghorn hens from a commercial strain were used in both experiments. The colostomized hens used in experiment 1 were prepared essentially according to the method of Ariyoshi and Morimoto (10). Separation of urine and feces was accomplished by collection of the urine in a collection bottle suspended with an appropriate harness, and fecal collection was made from a collecting pan beneath the metabolism cage in which the hens were housed. Chromic oxide was included in the experimental diets and nitrogen and energy digestibility were calculated by chromic oxide indicator method. Analysis of feed and feces for dry matter, nitrogen, chromic oxide and gross energy was made by procedures described by Anderson and Hill (11). Metabolizable energy values for diets in experiment 2 were also determined by procedures described by Anderson and Hill (11). The values reported are corrected to nitrogen equilibrium.

The colostomies were performed on the hens from 4 to 6 months prior to the experiment in which they were used, and the hens were approximately 13 months of age at the time of the experiment. All were in good condition prior to and during the experiment. The hens used in experiment 2 were just coming into peak production at the start of the experiment.

Unheated soybean meal used in these experiments was obtained as dehulled, hexane-extracted soybean flakes removed from a commercial soybean processing plant prior to desolventizing. The solvent was allowed to evaporate at ambient temperature. The heated meal in experiment 1 was prepared from the raw meal by autoclaving at 107° for 30 minutes under conditions described by Renner and Hill (8). In experiment 2, commercially produced 50% protein soybean meal was used as the heated meal control. The raw and heated meal were included in the diets to provide the same amount of dry matter.

RESULTS

In experiment 1, three colostomized hens were fed diet A shown in table 1 containing heated soybean meal and three were fed the same diet in which the soybean meal was unheated. After a few days' preliminary feeding, fecal collections

TABLE 1

Experimental diets

	A	B
	%	%
Soybean meal [1]	40.00	38.00
Soybean oil	2.00	3.00
Cornstarch	—	47.67
Sucrose	48.59	—
Cellulose	—	2.00
DL-Methionine	—	0.20
Dicalcium phosphate	3.30	3.30
Calcium carbonate	3.60	3.60
Sodium chloride	0.25	0.25
$ZnCO_3$	0.005	0.005
$MnSO_4$	0.03	0.03
Vitamin mixture [2]	1.00	0.75
Choline chloride, 70% solution	0.22	0.20
Chromium premix [3]	1.00	1.00

[1] Heated or unheated as indicated in the individual experiments.
[2] Same as described by Nesheim et al (14). In diet B, levels of vitamins were 75% of those in diet A.
[3] Contained 30% Cr_2O_3 in wheat flour.

TABLE 2

Digestibility of nitrogen and energy in diets containing heated or unheated soybean meal for colostomized hens

Treatment	Hen no.	Apparent nitrogen digestibility	Digestible energy/g diet DM	
			Determined	Corrected [1]
		%	*kcal*	*kcal*
Unheated meal	1	55.5	3.13	3.57
	2	52.7	2.93	3.40
	3	53.7	3.03	3.50
Average		54.0	3.03	3.49
Heated meal	4	83.6	3.38	3.46
	5	86.3	3.49	3.54
	6	87.1	3.56	3.60
Average		85.7	3.48	3.53

[1] Corrected to 90% protein digestibility — each gram of undigested protein in feces was assumed to have a gross energy of 5.65 kcal/g.

TABLE 3

Metabolizable energy value of diets containing raw or heated soybean meal for laying hens after various periods of feeding

Soybean meal treatment	Days fed diet [1]				Overall avg	Egg production [2]	Feed/ hen
	12	36	67	97			
	kcal/g dry matter				*kcal*	%	*kg*
Unheated	3.05	2.91	3.08	3.04	3.02	42	9.48
Heated	3.20	3.18	3.40	3.40	3.30	63	9.33

[1] Collections of excreta were begun on day indicated and proceeded for 4 consecutive days.
[2] Hen day production for 107 days.

were made for 3 consecutive days and nitrogen digestibility and digestible energy values were determined. The data obtained are shown in table 2. The nitrogen digestibility was markedly lower for hens fed the unheated soybean meal compared with those fed the heated soybean meal (54.0 vs. 85.7%). Variability between individual hens was very small. The digestible energy values were 3.48 and 3.03 kcal/g for the diets containing heated meal and unheated meal, respectively. By assigning a value of 5.65 kcal/g to the undigested protein that appears in the feces of hens receiving raw or heated meal, it was possible to calculate the digestible energy of the 2 diets when both were corrected to 90% protein digestibility. When this calculation was made the 2 diets had nearly identical amounts of digestible energy per unit of diet: 3.49 kcal/g for the hens receiving unheated soybean meal and 3.53 kcal/g for hens receiving heated soybean meal. This indicates that the main reason for the difference in digestible energy value between the 2 diets was the difference in protein digestibility.

Following the observations made in experiment 1, an experiment was designed to determine whether laying hens would adapt to prolonged feeding of unheated soybean meal.

In experiment 2 hens were fed diet B, table 1, containing heated or unheated soybean meal, for a period of 97 days. Metabolizable energy determinations were made after 12 days, 36 days, 67 days and 97 days of feeding the experimental diets. Records of egg production and feed consumption were also kept during the experimental period. Fecal collections were made for 4 consecutive days at each period, from 4 pairs of hens on each treatment.

The results of this experiment are shown in table 3. The metabolizable energy value of the diet containing raw soybean meal remained constant throughout the experimental period averaging slightly

over 3 kcal/g of diet. This was nearly the same as the digestible energy obtained for a similar diet in experiment 1. The diet containing heated meal was higher in metabolizable energy value in every case, ranging from 3.2 to 3.4 kcal/g of diet. Two different batches of heated meal were used during the experiment and one appeared to have a higher metabolizable energy value than the other. No change in the metabolizable energy value of the diet containing unheated soybean meal occurred over the experimental period. This suggests that the hens did not adapt to unheated soybean meal feeding in a way that resulted in better utilization of the unheated meal. Egg production was markedly lower in hens fed the diet containing unheated meal.

DISCUSSION

These experiments indicated that the utilization of unheated soybean meal by laying hens was very poor. The marked depression in protein digestibility observed is apparently sufficient to account for the difference in metabolizable energy value between unheated and heated soybean meal observed by Hill and Renner (7). The difference in digestible energy value of the diets used in this experiment could be completely explained by the differences in protein digestibility observed. The protein digestibility for unheated meal in this experiment was somewhat lower than that reported by Lepkovsky et al. (6) in studies with chickens with ileostomies (54% vs. 64 and 68%). However, these authors also reported that utilization of the non-nitrogenous portion of the diet was unaffected by consumption of unheated soybean meal.

In a recent paper, Nitsan (12) also has reported that digestibility of unheated soybean meal was low for cockerels, 3 to 4 months old, that were colostomized. Digestibility values reported in their study were similar to those reported in this paper.

In view of the results obtained in these studies, measurements of differences in metabolizable energy value of diets containing heated or unheated soybean meal for chicks or hens can probably be considered indirect measurements of differences in protein digestibility.

There was no evidence that the metabolizable energy value of the diet containing unheated soybean meal changed during a prolonged feeding period. Thus adaptations in intestinal proteolytic activity that have been shown by Nitsan and Alumot (5) may not reflect adaptation in actual utilization of unheated meal. In this respect these results would be consistent with the observations of Lepkovsky et al. (6) with adapted and unadapted chickens.

Most of the evidence reviewed above for adaptation to unheated soybean meal feeding has been based on the ability of diets containing unheated meal to support growth rate or egg production. No measurements of actual protein utilization were made. In view of the marked reduction in protein digestibility observed in this study, an examination of earlier results from this standpoint may be useful in explaining apparent discrepancies existing in the literature.

Fisher and Shapiro (13) reported that extra protein and energy could overcome most of the growth-depressing effects of feeding unheated soybean meal provided the studies were made on chicks over 3 weeks of age. Extra energy provided by fat to chicks under 3 weeks old fed unheated soybean meal is poorly utilized, since unheated meal inhibits fat absorption in young chicks (14).

Possibly some of the apparent adaptation to unheated soybean meal feeding has been due to the use of protein levels considerably above critical levels for the age studied.

Saxena et al. (2) observed that chicks 6 weeks of age or older grew much better than younger chicks when fed a diet containing about 25% protein from unheated meal. This protein level would be considerably less critical for the older than for the younger chickens. Probably up to one-third of the protein could then be indigestible without any depression of growth.

The same point could be made for the results of Bornstein and Lipstein (3), although somewhat lower levels of protein were used in their diets. The interpretation of the data in the experiments of these workers is based on the use of "percentage

gain" as the criterion, which does not appear to be valid, in our view. If "percentage gain" were not used, their data would be interpreted somewhat differently. Chicks at any comparable weight grew considerably more slowly when fed diets containing unheated rather than heated soybean meal.

The data of Fisher and Johnson (1) are also consistent with a lower protein digestibility for unheated soybean meal. These authors reported that to support egg production more protein was needed from unheated than from heated soybean meal.

Although the above discussion implies that poor digestibility of protein is the major reason for poor utilization of unheated soybean meal by chickens, other factors are probably also involved, particularly in affecting growth rate of young animals. Westfall et al. (15) showed that antitryptic preparations retarded growth rate of mice fed hydrolyzed protein. This could have been the result of poor digestion of endogenous protein, but other factors are probably also involved. Barnes et al. (16) have demonstrated an increased need for cystine in rats fed trypsin inhibitors, and Leiner (17) has demonstrated the detrimental effects of hemagglutinin concentrates on rat growth.

Another possible source of difference between experiments conducted with unheated soybean meal is the meal itself. We have observed marked differences in trypsin inhibitor content of batches of unheated soybean meal obtained from commercial sources. In particular, some samples of commercial soybean "brew" flakes have had only 25 to 50% of the trypsin inhibitor potency of unheated meal prepared by cold hexane extraction in our laboratory. It is likely that these variations in trypsin inhibitor content are related to effects of slight heat treatment in the production of the commercial products. Use of these different meals in feeding experiments might be expected to produce markedly different results.

There may also be other possible reasons for divergent results reported by other laboratories unrelated to those proposed above. There have been, however, sufficient reports where unheated soybean meal has not been well utilized by laying hens to make it unwise to consider the use of unheated soybean products in practical rations for adult chickens.

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Changes in Fatty Acid Composition in Liver Lipid Fractions of Pyridoxine-deficient Rats Fed Cholesterol [1]

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ABSTRACT This study was made to determine whether changes in fatty acid patterns of liver phospholipids and sterol esters accompanied the decreases in esterified sterol previously observed in pyridoxine-deficient rats. The diets included: (a) basal casein-5% cottonseed oil-sucrose diet; (b) basal diet plus cholesterol; (c) basal diet plus taurocholate; (d) basal diet plus cholesterol and taurocholate. Cholesterol was added to exaggerate the need for oleate and arachidonate since this stress might provide added information on unsaturated fatty acid metabolism in pyridoxine deficiency. Taurocholate was added to increase cholesterol absorption. These diets, with or without pyridoxine, were fed to male weanling rats for 6 weeks. Food intake of controls was restricted to the food intake of the corresponding deficient groups. Phospholipid tended to be lower in the deficient groups and liver sterol ester was significantly lower. All deficient groups showed a significant decrease in oleate in all lipid fractions, compared with their respective controls. Arachidonate and linoleate were significantly higher in sterol esters in all deficient groups. It was concluded 1) that pyridoxine deficiency significantly alters the metabolism of dietary and endogenous cholesterol and 2) that decreases in monoenoic acids in pyridoxine deficiency may be related to insulin insufficiency.

A low level of esterified sterol has been observed in pyridoxine-deficient rats showing an increased incorporation of acetate-^{14}C into liver sterol in vivo (1). In addition, pyridoxine-deficient rats fed cholesterol showed a smaller increase in liver cholesterol than pair-fed controls (2). The fatty acid composition of liver lipids, including the sterol esters, was not determined in these experiments.

Recent reports have indicated that pyridoxine deficiency increases the proportion of stearate and decreases the proportions of oleate and palmitoleate in total fatty acids of liver and adipose tissue in rats fed diets adequate in linoleate (3–5). Relatively little attention has been given to the effect of pyridoxine deficiency on the fatty acid composition of specific lipid classes, such as phospholipids and sterol esters. In the only study of this type, Swell et al. (6) reported an increase in the proportion of oleate and a decrease in arachidonate in liver phospholipid, which also decreased by 50%. Cholesterol esters showed no significant change in level or fatty acid pattern. Triglyceride, however, showed an increase in the proportion of stearate and a decrease in arachidonate.

The following experiment was carried out to determine whether any large changes in fatty acid pattern of liver phospholipids and sterol esters accompanied the decreased levels of esterified sterol previously observed in pyridoxine-deficient rats fed a 5% cottonseed oil diet (1). The dietary treatments included: (a) the basal casein-5% cottonseed oil-sucrose diet used previously (1); (b) the basal diet plus cholesterol; (c) the basal diet plus taurocholate; (d) the basal diet plus cholesterol and taurocholate. The cholesterol supplement was added to exaggerate the need for oleate and arachidonate in the metabolism of excess cholesterol (7, 8). Such a stress might provide added information on the metabolism of unsaturated fatty acids in pyridoxine deficiency. Dietary taurocholate was added to increase cholesterol absorption which

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might be impaired in the deficient rats because of decreased taurocholate secretion (9).[2]

METHODS

Male weanling rats of the Long-Evans strain, from the department colony (21 days and 45 to 55 g) were fed the experimental diets for 6 weeks. The rats were caged individually in suspended, galvanized, screen-wire cages. Tap water was given ad libitum. The dietary groups included: (a) the basal casein-cottonseed oil-sucrose diet;[3] (b) the basal diet plus 1.0% cholesterol; (c) the basal diet plus 0.5% sodium taurocholate; (d) the basal diet plus 1.0% cholesterol plus 0.5% sodium taurocholate. Cholesterol and taurocholate were substituted for an equal weight of sucrose. Each dietary group was subdivided into a pyridoxine-deficient and a pyridoxine-supplemented group. Pyridoxine deficiency was created by omission of pyridoxine from the vitamin supplement, without the use of pyridoxine antagonists. The food intake of each pyridoxine-supplemented control group was restricted to the average daily intake of the corresponding deficient group. The restricted-fed control rats were fed daily at 6 to 7 PM and ate all their food within one hour. On the last day of the experiment, the food cups were removed from both deficient and control rats at 8 PM. At 6 PM the next day, the rats were anesthetized with sodium pentobarbital[4] and killed by cardiac exsanguination with heparin as the anticoagulant. The livers were removed, weighed, lyophilized and the lipids extracted by the method of Tinoco et al. (11). Liver lipids were determined by the methods described by Scheier and Williams (12). Liver lipids were fractionated on silicic acid columns and the fatty acids determined by gas-liquid chromatography with methylheptadecanoate as the internal standard (13). Plasma sterol was determined in chloroform-methanol extracts (1:1) of the plasma. Differences between means are reported as significant at the 5% level (Student's *t* test).

RESULTS

Growth. In the first 3 weeks, the pyridoxine-deficient groups fed cholesterol or cholesterol plus taurocholate grew somewhat more than the other deficient groups (table 1). The highest mortality occurred in the deficient group fed the basal diet plus taurocholate although the survivors in this group showed the greatest gain of any of the deficient groups at the end of 6 weeks. Bloody feces and urine were observed in the deficient groups fed taurocholate or cholesterol and taurocholate, with a greater incidence in the former group. The control group fed taurocholate showed a greater incidence of diarrhea than the other control groups.

Liver. Liver phospholipid (mg/g) tended to be lower in the deficient groups than in the corresponding pair-fed controls although the decrease was significant only in the group fed the basal diet (table 2). The addition of either cholesterol or taurocholate to the basal diet did not affect phospholipid concentration, but the addition of both cholesterol and taurocholate significantly reduced the phospholipid level in both deficient and control groups.

Esterified sterol (mg/g) was significantly lower in all of the deficient groups, in comparison with their controls. Thus, feeding taurocholate with cholesterol did not increase liver sterol in the deficient rats to the level observed in the controls. Liver free sterol was slightly but significantly higher in all of the deficient groups.

The fatty acid composition of liver phospholipids is shown in table 3. All of the pyridoxine-deficient groups showed a significantly higher proportion of stearate and a significantly lower proportion of oleate, in comparison with their controls. Linoleate differed only in the deficient group fed the basal diet, in which the proportion was significantly higher. The proportion of arachidonate was not re-

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[2] Kelly, R. L., and E. A. Doisy, Jr. 1964 Descending thin layer chromatography (TLC) for assay of whole bile. Federation Proc., 23: 173 (abstract).

[3] Composition of basal diet, g/100 g diet: vitamin-free casein (Nutritional Biochemicals Corporation, Cleveland), 20.0; cottonseed oil, 5.0; Jones-Foster salts (10) (General Biochemicals, Inc., Chagrin Falls, Ohio), 4.0; choline bitartrate, 0.18; B vitamin premix in sucrose, 2.0; vitamin A, D, E mix in cottonseed oil, 1.0; powdered sucrose, 67.82. The vitamin mixes provided the following levels of vitamins, μg/g diet: thiamine·HCl, 10; riboflavin, 20; niacinamide, 120; Ca D-pantothenate, 64; pyridoxine·HCl (when added), 20; folic acid, 4; biotin, 2; vitamin B_{12}, 0.04; menadione, 0.8; vitamin A, 10 IU; vitamin D, 1 IU; DL-α-tocopheryl acetate, 0.22 IU.

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

TABLE 1

Effect of diet on weight gain

Diet [1]	Vitamin B_6	Initial wt	Weeks fed diet: 1	2	3	4	5	6	Mortality	Avg 6-week food intake
			Average cumulative weight gain							
		g	*g*	*g*	*g*	*g*	*g*	*g*		*g*
Basal	−	53.3	16	26	28	32	33	30	0/11	273
	+	47.1	22	45	52	63	72	80	0/9	
Basal + C	−	49.9	17	30	38	42	46	43	0/11	253
	+	45.5	14	34	42	50	58	62	0/10	
Basal + T	−	51.0	14	24	28	48 [2]	55	58 [2]	4/11	322
	+	47.4	18	45	59	75	90	103	0/10	
Basal + C + T	−	49.2	14	24	33	35	41 [3]	40	2/11	277
	+	45.2	13	34	48	60	69	76	0/10	

[1] C indicates cholesterol; and T, sodium taurocholate.
[2] One death in fourth week; 3 deaths in sixth week.
[3] Two deaths in fifth week.

TABLE 2

Liver lipids

Diet [1]	Vitamin B_6	No. of rats	Liver wt	Phospholipid	Sterol: Total	Free	Ester
			g	*mg/g*	*mg/g*	*mg/g*	*mg/g*
Basal	−	11	2.62	30.3 ± 1.4 [2]	3.30 ± 0.17	2.71 ± 0.14	0.59 ± 0.05
	+	10	3.92	35.4 ± 0.4	2.81 ± 0.09	1.95 ± 0.04	0.85 ± 0.11
Basal + C	−	9	3.06	32.9 ± 1.2	11.3 ± 4.2	2.61 ± 0.15	8.69 ± 4.20
	+	9	4.08	34.0 ± 0.9	24.8 ± 3.0	2.28 ± 0.08	22.50 ± 2.99
Basal + T	−	7	3.59	31.4 ± 1.6	4.15 ± 1.71	2.65 ± 0.18	1.50 ± 0.17
	+	10	5.27	34.4 ± 0.6	4.47 ± 0.29	2.11 ± 0.04	2.36 ± 0.28
Basal + C + T	−	9	4.03	25.9 ± 1.7	80.6 ± 5.9	4.85 ± 0.37	75.7 ± 5.9
	+	10	6.75	27.3 ± 1.0	118.3 ± 6.0	3.35 ± 0.21	115.0 ± 5.9

[1] C indicates cholesterol; and T, sodium taurocholate.
[2] Mean ± SE.

duced and was even significantly higher in the deficient groups fed cholesterol or cholesterol plus taurocholate, in comparison with their controls. The proportion of total unsaturated fatty acids remained constant in the deficient groups since the lower oleate levels were counterbalanced by the increases in linoleate or arachidonate. In terms of milligrams/liver, the amounts of all fatty acids were lower in the deficient groups because of the smaller liver size and the tendency toward lower phospholipid levels.

In sterol esters (table 4), the proportion of oleate was significantly lower and the proportions of linoleate and arachidonate significantly higher in the deficient groups. The proportion of palmitoleate was significantly lower in all deficient groups except the one fed the basal diet. The proportion of total unsaturated fatty acids stayed relatively unchanged since the lower oleate values were counteracted by the higher linoleate and arachidonate values.

In liver triglyceride, the proportion of oleate was significantly lower in the deficient groups than in the corresponding controls (table 5). Stearate tended to be higher, but the increase was statistically significant only in the deficient group fed cholesterol. Because of the decrease in oleate, the proportion of total unsaturated fatty acids was lower except for the deficient group fed cholesterol and taurocholate. In this group, the total unsaturated fatty acids remained constant because of the large increase in linoleate.

TABLE 3

Proportions of fatty acids in liver phospholipids

iet [1]	Vitamin B_6	No. of rats	Liver wt	Phospholipid	Fatty acids							Unsaturated fatty acids	
					14:0	16:0	16:1	18:0	18:1	18:2	20:4	Total	Monoenoic
			g	*mg/g*	wt %	wt %	wt %	wt %	wt %	wt %	wt %	%	%
al	−	8	2.66 ± 0.18 [2]	30.1 ± 1.8	0.2 ± 0.1	16.3 ± 1.0	0.6 ± 0.1	28.8 ± 1.0	5.6 ± 0.3	17.0 ± 0.7	31.4 ± 1.5	54.6	6.2
	+	6	4.00 ± 0.14	35.4 ± 0.5	0.2 ± 0.1	19.0 ± 0.7	0.6 ± 0.3	23.9 ± 0.7	9.0 ± 0.6	13.4 ± 0.7	33.8 ± 0.5	56.8	9.6
al + C	−	6	2.95 ± 0.18	32.2 ± 1.8	0.1 ± 0.1	14.6 ± 0.6	0.5 ± 0.1	29.0 ± 0.4	5.7 ± 1.0	17.0 ± 1.0	33.2 ± 1.0	56.4	6.2
	+	6	4.01 ± 0.26	34.3 ± 1.0	0.2 ± 0.1	17.8 ± 0.6	1.6 ± 0.1	22.5 ± 0.4	10.8 ± 0.4	17.0 ± 0.4	29.4 ± 0.7	58.8	12.4
al + T	−	6	3.58 ± 0.25	31.0 ± 1.9	0.3 ± 0.1	16.6 ± 0.8	0.6 ± 0.1	27.4 ± 0.8	6.2 ± 0.4	14.3 ± 0.5	34.6 ± 0.5	55.7	6.8
	+	6	5.36 ± 0.24	34.4 ± 0.8	0.2 ± 0.1	19.7 ± 0.3	1.2 ± 0.1	22.4 ± 0.7	10.0 ± 0.4	13.2 ± 0.5	33.1 ± 0.5	57.5	11.2
al + C + T	−	6	3.96 ± 0.48	24.8 ± 2.4	0.2 ± 0.1	15.0 ± 2.2	1.0 ± 0.1	28.4 ± 1.0	8.2 ± 0.4	17.8 ± 0.6	29.4 ± 0.9	56.4	9.2
	+	6	6.85 ± 0.22	28.1 ± 1.0	0.3 ± 0.1	18.3 ± 0.3	1.8 ± 0.2	21.9 ± 1.0	12.8 ± 0.8	18.4 ± 0.9	25.7 ± 1.2	58.7	14.6

C indicates cholesterol; and T, sodium taurocholate.
Mean ± SE.

TABLE 4

Proportions of fatty acids in liver sterol esters

Diet [1]	Vitamin B_6	No. of rats	Liver wt	Ester sterol	Fatty acids							Unsaturat fatty acic	
					14:0	16:0	16:1	18:0	18:1	18:2	20:4	Total	Mo en
			g	*mg/g*	wt %	wt %	wt %	wt %	wt %	wt %	wt %	%	%
sal	−	4	2.59 ± 0.26 [2]	0.6 ± 0.10	ND [3]	21.8 ± 1.4	3.2 ± 0.8	21.2 ± 2.8	13.6 ± 3.9	21.9 ± 2.4	18.2 ± 2.2	56.9	16.
	+	6	4.00 ± 0.14	0.95 ± 0.20	0.4 ± 0.1	23.6 ± 0.7	4.0 ± 0.8	19.8 ± 4.4	29.6 ± 4.1	14.2 ± 1.1	8.4 ± 2.0	56.2	33.
sal + C	−	5	2.82 ± 0.14	1.76 ± 0.60	0.5 ± 0.1	17.3 ± 4.8	3.7 ± 0.2	11.3 ± 1.2	31.2 ± 2.1	24.7 ± 1.1	11.1 ± 2.1	70.7	34.
	+	6	4.02 ± 0.26	23.91 ± 4.00	0.5 ± 0.1	15.7 ± 0.3	10.4 ± 0.2	3.9 ± 0.2	50.6 ± 0.9	16.5 ± 0.6	1.5 ± 0.5	79.0	61.
sal + T	−	6	3.58 ± 0.25	1.43 ± 0.18	0.2 ± 0.2	15.2 ± 0.6	3.0 ± 0.4	11.9 ± 2.5	31.2 ± 2.5	23.8 ± 1.8	14.3 ± 1.2	72.3	34.
	+	6	5.41 ± 0.24	2.80 ± 0.29	0.2 ± 0.1	17.2 ± 1.0	5.7 ± 0.6	7.7 ± 0.7	47.0 ± 1.6	17.1 ± 1.1	5.1 ± 0.6	74.9	52.
sal + C + T	−	6	3.96 ± 0.48	81.8 ± 7.4	0.3 ± 0.2	13.6 ± 0.7	7.0 ± 0.8	2.9 ± 0.4	41.6 ± 0.6	29.8 ± 1.1	3.6 ± 0.8	82.0	48.
	+	5	6.89 ± 0.27	110.6 ± 10.5	0.4 ± 0.1	13.9 ± 0.5	12.0 ± 0.3	2.3 ± 0.2	51.9 ± 0.9	18.1 ± 0.4	ND	82.0	63.

[1] C indicates cholesterol; and T, sodium taurocholate.
[2] Mean ± SE.
[3] Not detected.

TABLE 5

Proportions of fatty acids in liver triglycerides

Diet [1]	Vitamin B_6	No. of rats	Liver wt	Fatty acids	Fatty acids							Unsaturated fatty acids	
					14:0	16:0	16:1	18:0	18:1	18:2	20:4	Total	Monoenoic
			g	*mg/g*	wt %	wt %	wt %	wt %	wt %	wt %	wt %	%	%
ısal	—	6	2.71 ± 0.23 [2]	0.86 ± 0.64	0.2 ± 0.1	36.9 ± 2.1	1.4 ± 0.5	28.3 ± 7.3	20.1 ± 3.5	13.1 ± 5.1	ND [3]	34.6	21.5
	+	6	4.00 ± 0.14	1.27 ± 0.37	0.5 ± 0.1	35.9 ± 1.5	2.2 ± 0.5	10.4 ± 5.0	39.9 ± 2.1	10.7 ± 0.9	0.3 ± 0.2	53.1	42.1
ısal + C	—	6	2.95 ± 0.18	5.57 ± 4.38	0.5 ± 0.1	30.9 ± 2.5	2.2 ± 0.3	24.5 ± 7.7	19.3 ± 2.0	20.7 ± 6.8	3.2 ± 1.6	45.4	21.5
	+	5	3.85 ± 0.24	4.82 ± 2.12	0.6 ± 0.1	27.2 ± 1.3	3.2 ± 0.3	5.1 ± 1.0	44.4 ± 2.0	19.3 ± 3.3	ND	66.9	47.6
ısal + T	—	6	3.58 ± 0.25	0.65 ± 0.36	1.2 ± 0.7	30.9 ± 4.1	3.8 ± 1.4	15.4 ± 5.8	23.7 ± 2.9	19.4 ± 1.7	5.0 ± 1.7	51.9	27.5
	+	6	5.36 ± 0.24	2.46 ± 0.25	0.7 ± 1.0	28.0 ± 1.7	3.3 ± 0.2	5.6 ± 0.6	41.2 ± 1.8	19.4 ± 1.3	1.1 ± 0.6	64.5	44.5
ısal + C + T	—	6	3.96 ± 0.48	4.46 ± 1.22	0.6 ± 0.2	18.7 ± 1.5	3.0 ± 1.2	6.9 ± 1.5	32.8 ± 1.4	33.4 ± 3.5	2.7 ± 1.0	71.9	35.8
	+	5	6.89 ± 0.27	6.89 ± 0.40	0.5 ± 0.1	21.4 ± 0.2	3.3 ± 0.2	3.9 ± 0.2	45.9 ± 1.0	23.3 ± 0.3	1.3 ± 0.9	73.8	49.2

[1] C indicates cholesterol; and T, sodium taurocholate.
[2] Mean ± SE.
[3] Not detected.

Plasma. The addition of cholesterol to the diet increased plasma sterol levels in both the deficient and control groups, with greater increases with the addition of both cholesterol and taurocholate (table 6). However, total sterol in all of the deficient groups was lower than in the corresponding controls although the difference was significant only when cholesterol was added to the diet. Thus, pyridoxine deficiency, under these conditions, did not produce hypercholesterolemia.

DISCUSSION

Fatty acid composition. The phospholipids of the deficient rats fed the basal diet showed a significant increase in stearate and linoleate, a significant decrease in oleate, and no change in arachidonate. Sterol esters showed significant increases in linoleate and arachidonate and a significant decrease in oleate. These results contrast with those of Swell et al. (6). Possible reasons for the differences include the composition of the diets and the degree of pyridoxine deficiency. The diet of Swell et al. (6) had higher levels of protein and fat and a different source of carbohydrate (27% casein, 10% vegetable oil and 59% starch). Furthermore, under their conditions, the deficient rats showed a much larger decrease (50%) in liver phospholipids than those of the present experiment. This may be responsible for the large decrease in phospholipid arachidonate.

The results with all of the deficient groups in the present experiment, however, are consistent with previous reports of an increase in the proportion of stearate and a decrease in the proportion of monoenoic acid in carcass and liver total fatty acids of pyridoxine-deficient rats (3–5). All 4 deficient groups showed a significant increase in the proportion of stearate in phospholipid and sterol ester, and a significant decrease in oleate in all lipid classes. In three of the four deficient groups, there was also a decrease in palmitoleate in phospholipid and sterol ester. Thus, changes in stearic and monoenoic acids appear to occur more frequently in pyridoxine deficiency than changes in arachidonate, at least with rats fed diets adequate in linoleate. It is reasonable to expect that arachidonate levels would be less affected in experiments where pyridoxine deficiency is developed with diets adequate in linoleate than in experiments testing the effect of pyridoxine deficiency on linoleate utilization in recovery from essential fatty acid depletion (12, 14). In the former type of experiment, growth is decreasing or has stopped so that less arachidonate is needed for synthesis of new tissue. Hence a longer time might be required to show any effects on arachidonate levels since the need for arachidonate has been reduced and since considerable formation of arachidonate occurs even in rats severely deficient in pyridoxine (12, 14). However, in experiments testing the effect of pyridoxine deficiency on recovery from essential fatty acid depletion, any effects of pyridoxine deficiency on arachidonate metabolism

TABLE 6

Plasma sterol levels

Diet [1]	Vitamin B_6	No. of rats	Sterol		
			Total	Free	Ester
			mg/100 ml	*mg/100 ml*	*mg/100 ml*
Basal	–	9	62 ± 4 [2]	16 ± 2	46 ± 11
	+	9	68 ± 4	19 ± 1	49 ± 4
Basal + C	–	9	80 ± 5	20 ± 1	60 ± 4
	+	7	121 ± 8	25 ± 2	96 ± 7
Basal + T	–	6	68 ± 4	17 ± 1	50 ± 3
	+	9	73 ± 3	19 ± 2	54 ± 4
Basal + C + T	–	7	109 ± 14	26 ± 2	93 ± 10
	+	8	187 ± 18	31 ± 3	156 ± 4

[1] C indicates cholesterol; and T, sodium taurocholate.
[2] Mean ± SE.

should be more pronounced because of the greater need for formation of arachidonate (or lipids containing arachidonate) in animals recovering from essential fatty acid (arachidonate) deficiency.

The decrease in monoenoic fatty acid in pyridoxine deficiency could result from increased utilization as well as decreased synthesis. There is as yet no evidence for increased utilization. There is some evidence, however, to support the idea of decreased synthesis. Huber et al. (5) reported that treatment with insulin prevented the increase in stearate and the decrease in palmitoleate in total fatty acids of liver and adipose tissue of pyridoxine-deficient rats. These authors suggested that the changes in fatty acid composition, as well as other symptoms in the deficient rats, could be explained by a decreased availability of insulin. Recently, Benjamin and Gellhorn (15) reported an increase in the proportion of stearate and a decrease in the proportions of palmitoleate and oleate in the adipose tissue of alloxan-diabetic rats. Conversion of stearate to oleate was also reduced in microsomal preparations from liver or adipose tissue of diabetic rats (16). Consequently, if insulin insufficiency occurs in pyridoxine-deficient rats, then the synthesis of palmitoleate and oleate might decrease since insulin may be necessary for these reactions to occur at a normal rate. Further study is needed on the relationships between insulin insufficiency and the symptoms of pyridoxine deficiency.

Phospholipid levels. The tendency to lower phospholipid values in the deficient groups supports the idea of altered phospholipid metabolism (6, 12). Changes in the proportions of specific phospholipid fractions (17) could underlie the changes observed in the proportions of phospholipid stearate and oleate in the deficient rats. Phospholipid synthesis could be decreased by pyridoxine deficiency in several ways. The synthesis of phospholipid bases depends quite directly on pyridoxal-phosphate catalyzed reactions (18). Pyridoxine deficiency could also interfere with the synthesis of glycerol-phosphate and lipoproteins, both of which are needed for phospholipid synthesis in microsomes (19, 20). Glycerol-phosphate formation could be reduced if gluconeogenesis from amino acids were reduced as a result of decreased transaminase activity in pyridoxine deficiency (21). Lipoprotein synthesis could be reduced if the synthesis and interconversion of amino acids (18) by pyridoxal-phosphate reactions were reduced by the deficiency to an extent that the availability of specific amino acids limited the rate of lipoprotein synthesis. Furthermore, all of these reactions could also be affected by any changes in the production of insulin or pituitary and adrenal hormones in the deficient rats (5).

Liver sterol. Liver esterified sterol in the deficient group fed the basal diet was lower and liver-free sterol slightly higher than in the controls. In other cases,[5] however, significant differences have not been observed in deficient rats fed the basal diet although free sterol tends to be higher and esterified sterol lower in the deficient rats. These trends in deficient rats not fed cholesterol are in contrast with other results (6) indicating a significant decrease in liver-free cholesterol with no change in esterified sterol.

The changes observed in the present experiment, especially in the groups fed cholesterol, could reflect changes in liver metabolism or changes in absorption, or both. Changes in liver metabolism are indicated by the significantly higher proportions of linoleate and arachidonate and the significantly lower proportion of oleate in sterol esters in all of the deficient groups. The reasons for these significant alterations in the pattern of liver sterol esters remain to be investigated.

Cholesterol absorption might be decreased if pyridoxine deficiency reduced the level of cholesterol esterase in the intestine (22). This possibility remains to be tested. Decreased taurocholate secretion in pyridoxine deficiency (9)[6] does not appear to be a major cause of the lower esterified sterol in the deficient groups. This is indicated by the fact that feeding cholesterol with taurocholate did not raise the level of esterified sterol to the level of the control group with the same intake of cholesterol and taurocholate.

[5] Unpublished results, M. A. Williams.
[6] See footnote 2.

Plasma sterol. All of the deficient groups showed a lower plasma sterol level. This is in contrast with other reports of no change or an increase in plasma sterol in pyridoxine-deficient rats. Swell and coworkers (6) observed no difference in esterified or free sterol between deficient and control rats fed a diet containing 10% vegetable oil, without added cholesterol. Goswami and Sadhu (23) reported hypercholesterolemia in pyridoxine-deficient rats fed a diet containing 9% coconut oil, without added cholesterol. Shue and Hove (24) reported hypercholesterolemia in deficient male rats with 20% coconut oil or cottonseed oil, 3% cholesterol and 1% cholic acid, but not with lard as the dietary fat. These divergent observations show that no generalization can be made concerning the effect of pyridoxine deficiency on plasma cholesterol since the effect depends upon the conditions under which the deficiency has been produced.

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Effect of Low-protein Diet on the Ability of the Adult Rat to Recover from a Sublethal Dose of Irradiation [1]

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ABSTRACT These data support the conclusion that a low protein diet fed to adult rats after a sublethal dose of irradiation did not affect repair processes. Those end points which are affected by the low protein diet per se — serum albumin and liver protein metabolism as indicated by liver protein/DNA, RNA/DNA ratios, methionine uptake and amino acid content — are also lower for the irradiated animals fed 5% casein than for the irradiated animals fed 15% casein. These effects are not compounded by the irradiation, and in fact, irradiation appeared to serve as a stimulus in the 5% casein group, maintaining liver protein metabolism immediately after irradiation at a level higher than that of the 5% casein-fed controls and equal to that of controls fed 15% casein. However, the detrimental effects of a low protein diet were observed in both control and irradiated rats, and in this respect it is probable that a low protein diet is of no actual benefit following irradiation. In conclusion, when an adult rat had an adequate protein reserve, the feeding of a low protein diet after irradiation did not inhibit its recovery.

The nutritional status of an animal appears to have variable effects on the recovery from radiation injury. Cornatzer (1) reported that rats fed a low protein (10%), low fat (5%) diet following injection of a lethal dose of ^{32}P survived longer than those in which either or both of these dietary components were increased. It has also been reported that rats that were given small amounts of ethyl linoleate or methyl linoleate when kept on a low fat diet after irradiation survived longer than those that did not receive this supplement (2, 3).

It is well known that for the first few days following irradiation in the LD_{50} range, rats eat little, if anything. Considering the possibility that this self-imposed fasting might be detrimental, Smith et al. (4) studied the effects of force-feeding during this period. In rats force-fed a protein hydrolysate dextrose mixture, body weight was maintained, following exposure to 800 r. However, this dose of irradiation resulted in 100% mortality of force-fed animals, whereas only 80% of the ad libitum controls died. Force-feeding of fat has also been reported to be detrimental to irradiated animals (5). Furthermore, increasing body weight by injecting gold thioglucose prior to irradiation had no beneficial effect. Similarly a genetically fatter strain of rats was no more resistant to irradiation (6).

However, a number of investigations have shown that protein depletion of the animal before exposure to irradiation may be harmful to the recovery process. Feeding rats a low protein diet until they lost 25% of their body weight reduced the $LD_{50/30}$ value from 700 r to 520 r (7). A less severe depletion — a low protein diet for 3 weeks prior to irradiation — was not as detrimental to survival of rats exposed to 550 r and had no effect on those given 450 r (8).

The above investigations suggest that the composition of diet fed after irradiation did not markedly influence the recovery process. However, severe protein depletion prior to irradiation reduced the

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survival rate. The concept of protein reserve (9) may provide the explanation of these observations. During protein depletion, the animal loses its protein reserves (9) and demonstrates decreased ability to respond to stress (10). The adult rat fed an adequate diet prior to irradiation apparently can utilize its protein reserve to help in the recovery from irradiation damage. Mobilization of the protein reserves can be characterized by loss in body weight, negative nitrogen balance, flow of amino acids from muscle and skin to viscera, and increase in concentration of cellular liver protein, RNA and free amino acids (11–13). Mortality, which has been used in most studies on the effect of nutrition on recovery from ionizing radiation, provides a limited amount of information. Therefore, in the present study, various biochemical parameters were investigated in an attempt to gain insight into the problem of the interaction between nutritional state of an animal and its response to a sublethal dose of irradiation.

MATERIALS AND METHODS

Experimental regimen of animals fed an adequate diet. Forty male rats of the Wistar strain (group A), with an average body weight of 350 to 400 g, were placed in individual plastic cages for 16 hours, during which time they received a total dose of 350 r of γ-radiation from a ^{60}Co source. Prior to, during, and post-irradiation these rats were fed an agar-gel diet (10) ad libitum. The diet consisted of the following: (in per cent of dry diet) casein protein, 15; carbohydrate (as sucrose, dextrose, and dextrin in a ratio of 1:1.58:1.04), 51.6; lard, 24; salt mixture (14), 4; agar, 3.3; cod liver oil, 0.2; and water, 140; also the following vitamins per kilogram of dry diet: (in milligrams) thiamine, 10; riboflavin, 20; pyridoxine, 10; menadione, 15; niacin, 80; pantothenic acid, 80; inositol, 200; folic acid, 0.5; biotin, 0.5; *p*-aminobenzoic acid, 80; choline, 2000; ascorbic acid, 2; and α-tocopherol, 100. The water, lard and agar were heated until the solid material had melted. This solution was added to the dry ingredients and mixed. The vitamins were added when the mixture had cooled but was still fluid. This agar-gel diet aids in the accuracy of measuring food consumption.

Another group (B) of 16 rats which served as controls, was treated exactly the same as group A, but was not exposed to any radiation. At zero, 3, 7, 14, and 28 days after irradiation 8 animals from group A were autopsied, and 8 rats in group B were killed at zero and 28 days. One hour before necropsy 5 μc of ^{35}S-DL-methionine were injected intraperitoneally into each animal. All rats were anesthetized with pentobarbital (60 mg/kg) and killed by exsanguination. Samples of blood were taken for the determination of red blood count, white blood counts, hematocrit, and serum protein electrophoresis and the livers were removed and saved for chemical analysis.

Experimental regimen of animals fed a low protein diet. A third group (C) of 32 male rats of the Wistar strain with an average weight of 350 to 400 g were placed in plastic cages and irradiated as above, whereas group (D) (32 animals) served as non-irradiated controls. Prior to irradiation, all rats were fed the 15% casein protein agar-gel diet. During and after irradiation, all animals were fed the agar-gel basal diet; however, the casein protein content was reduced to 5% and carbohydrate was increased to 61.6%, as sucrose, dextrose and dextrin were added in a ratio of 1:1.58:1.04.

At 3, 14, and 28 days after irradiation, 8 animals from each group were killed. All rats were injected one hour before autopsy with 5 μc of ^{35}S-DL-methionine, and at necropsy blood and liver samples were taken for biochemical analysis.

All animals were housed at a temperature of 26 $\pm$ 1° with a relative humidity of 40%. Animal quarters were illuminated daily from 6 AM to 6 PM. Daily food intakes and weekly body weights were recorded for each animal throughout the experiment.

Analyses. The livers were homogenized individually with ice-cold distilled water into a 20% suspension and the total protein concentration, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and radioactive ^{35}S protein were determined (15). The free amino acids were determined by ion exchange chromatography (16) on the

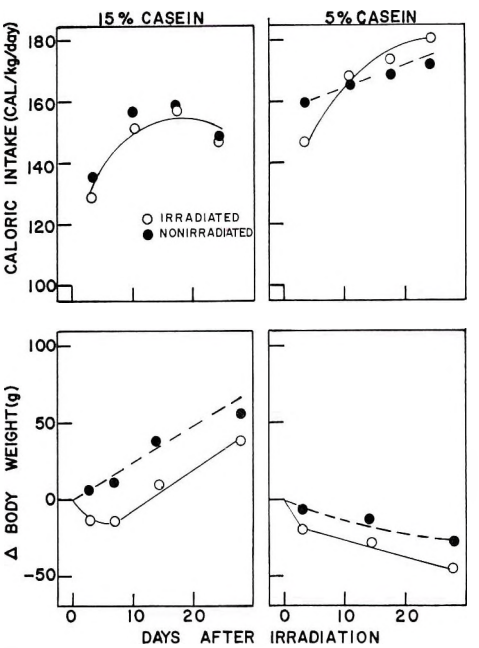

Fig. 1 Effects of dietary protein on change in body weight and caloric intake are plotted at various times after exposure to γ-radiation. Open circles are rats exposed to 350 r of radiation and solid circles, non-irradiated controls. Caloric intakes are plotted as mean weekly intakes. Each value is the mean of 8 animals.

picric acid filtrate of pooled aliquots from each group. Serum from each animal was separated into various component protein fractions (17) by paper electrophoresis. Red and white cell counts were determined in a Coulter counter and hematocrits were measured by the micro-technique of Strumia et al. (18).

Standard errors were determined for all values. If the probability value was less than 0.01, the difference between the means was considered significant.

EXPERIMENTAL RESULTS

The effects of irradiation on caloric intake and body weight gain are shown in figure 1. For the first few days following irradiation, the caloric intake decreased; but by the end of the first week the animals resumed normal feeding. Body weight of the rats fed both the 5% and 15% casein diets showed an initial decrease. However, in the 15% group, body weight returned to the control level by the end of the experiment, whereas in the 5% group, weight was lost throughout duration of the experiment. Following the initial decrease the rate of weight loss was the same as the controls during the last 3 weeks.

Figure 2 shows the white and red cell counts. In both groups, red cell counts decreased steadily, reaching their lowest levels during the second week and returning to control values by the end of the experiment. White cell counts decreased rapidly to very low levels in both groups, remaining low during the first week and recovering to control values by the end of the fourth week. Control values were unaffected by the diet. Hematocrit values were not affected by radiation.

Irradiated rats fed a 15% casein diet showed an early decrease in total globulin and γ-globulin values which slowly returned to normal by the end of the experiment (fig. 3). Serum albumin in this group also decreased sharply during the

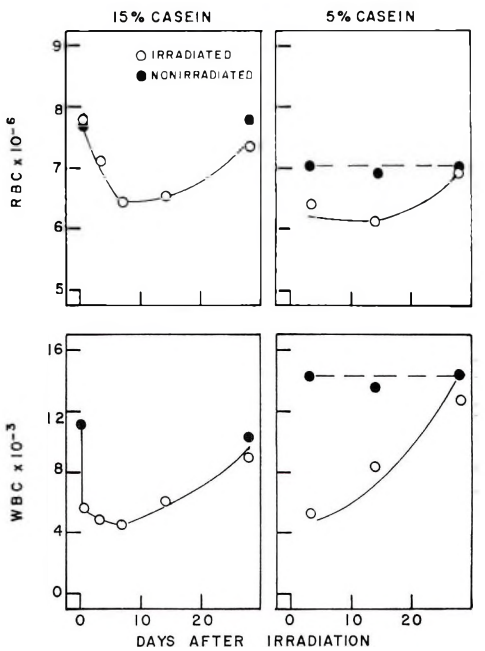

Fig. 2 Effects of dietary protein on white blood count (WBC) and red blood counts (RBC) are plotted at various times after exposure to γ-radiation. For 5% casein the plot is started at 3 days, the first experimental and control groups that were removed. Each value is the mean of 8 animals.

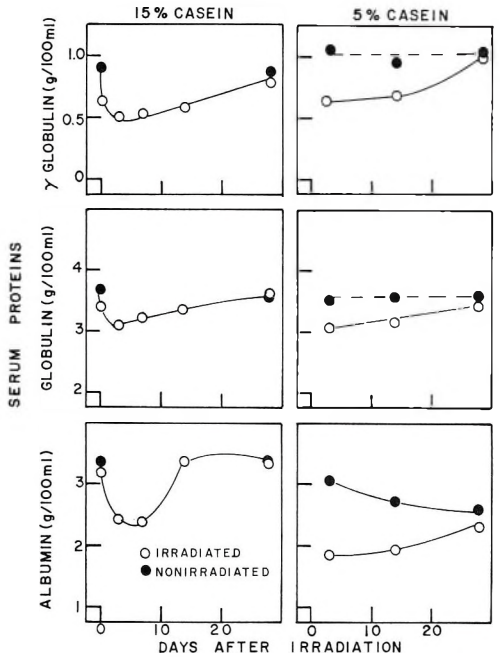

Fig. 3 Effects of dietary protein on the concentration of serum albumin, total globulin and γ-globulin are plotted at various times after exposure to γ-radiation. Each value is the mean of 8 animals.

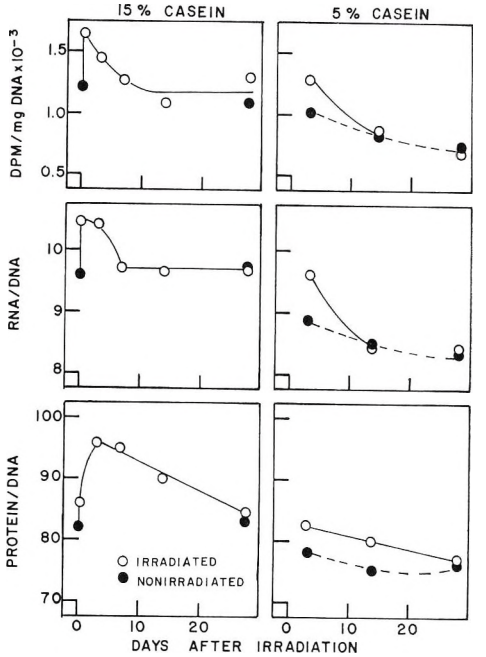

Fig. 4 Effects of dietary protein on the liver protein/DNA (mg/mg) RNA/DNA (mg/mg) and uptake of ^{35}S from DL-methionine are plotted at various times after exposure to γ-radiation. Each value is the mean of 8 animals.

first week, but returned to the control level by the end of the second week. In the 5% casein group, serum protein values decreased to below control values 3 days after irradiation and recovered slowly to control values by the end of the experiment. The 5% casein diet fed to the unirradiated animals did not affect serum globulin values but did result in a steady slow decline in serum albumin values.

Figure 4 shows some aspects of the irradiation and diet effect on liver metabolism. Protein/DNA ratios, RNA/DNA ratios and uptake of ^{35}S-methionine into liver protein all increased in the irradiated animals fed 15% casein in the first 3 days, and during the course of the experiment returned to control values. In the irradiated animals fed 5% casein, these values also increased above control values 3 days after irradiation. They returned to control levels during the second week in the case of the RNA/DNA ratios and uptake of ^{35}S-methionine, and by the end of the fourth week in the case of the protein/DNA ratios. In the unirradiated controls fed 5% casein, these end points decreased slowly throughout the course of the experiment.

In the irradiated animals fed 15% casein, there was an immediate sharp increase in both essential and nonessential amino acids in the liver (fig. 5). The former returned to control levels by the end of the first week, the latter by the end of the second week. In the irradiated animals fed 5% casein, amino acid levels were elevated over control values 3 days after irradiation and returned to control levels during the second week. In the unirradiated controls fed 5% casein, liver amino acid levels decreased slowly throughout the experiment.

DISCUSSION

Loss of appetite, the duration of which is proportional to the amount of irradiation, is characteristic of rats that are exposed to irradiation (19), a transient decrease in appetite being reported follow-

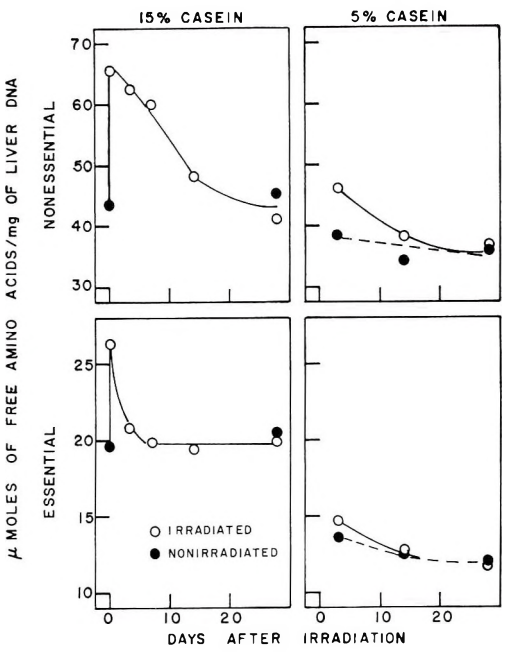

Fig. 5 Effects of dietary protein on the concentration of liver essential and nonessential free amino acids are plotted at various times atfer exposure to γ-radiation. Each value is the mean of 8 animals.

ing doses as low as 50 r (20). In the present experiment, caloric intake decreased during the first 3 days. The rats, however, did eat during the immediate post-irradiation period, although not as much as they would have normally. The body weight loss observed during the first week in the animals fed 15% casein cannot be explained completely by the decreased food intake and may be the result of tissue breakdown following the radiation insult. In the 5% casein groups, both control and irradiated animals lost weight at the same rate following the initial rapid loss experienced by the irradiated animals during the first 3 days. The body weight in the irradiated animals thus never returned to control level.

The decrease in the red and white cell counts observed in the present experiment is characteristic of radiation injury, white cell counts decreasing more rapidly and to lower values than red cell values (21, 22). The pattern of the decline and the recovery of both the red and white cells in the peripheral blood (fig. 2) agrees completely with that reported for rats exposed to 300 r of x-irradiation (23). Although a decrease in red cell count, apparently due to lack of erythropoieten, has been reported in animals fed a portein-free diet (24), the control rats in the present study fed 5% casein did not show either this decrease or any effects on the white cell count. The irradiated animals fed 5% casein show the decrease in red and white cell counts and they also show the recovery to control levels by the end of the experiment. Thus, even when rats are maintained with a low protein diet, the radiation damage to the hematopoietic system is repaired just as rapidly and just as completely as in animals fed a diet with normal protein levels.

Serum proteins show approximately the same pattern in both the 5% and 15% groups (fig. 3). The curves indicate that the slight decrease in total globulin immediately following irradiation is due mainly to a decrease in γ-globulin. The low protein diet had no effect on the serum globulin, and in addition, despite the diet, the irradiated rats showed a return of serum globulin values to normal just as rapidly as the value for the rats fed 15% protein. Serum albumin, however, decreased slowly in the unirradiated controls fed 5% casein. This is characteristic of animals maintained with a low protein diet (25). At the same time that control values were decreasing, however, those of irradiated rats fed 5% casein were increasing and reached control levels by the end of the fourth week. The irradiation damage reflected in the initial decrease in serum albumin values is thus repairable despite the low protein diet.

Immediately after irradiation cellular RNA and essential free amino acid of the liver were elevated above the values noted in control animals. The higher concentration of these cellular constituents is reflected in an increased rate of protein biosynthesis and subsequent elevated concentration of cellular protein. These changes in liver composition are correlated with a loss in body weight and lower serum albumin concentration. Similar tissue responses have been noted in other stress conditions such as tumor cachexia (11), infection (26), injury (27), neph-

ritis (28), pregnancy (29), lactation (30), and adrenal corticoid administration (12). All of these stress responses are characterized by loss of muscle and skin protein resulting in a flow of amino acids away from the muscle and skin toward the viscera, negative nitrogen balance of the carcass and an increase in liver protein, RNA and free amino acids (11). These changes are apparently a homeostatic response whereby the protein reserves of the body supply amino acids for the rapid repair of the harmful effects of stress.

As reported previously (31), feeding a low protein diet results in a decreased concentration of liver protein, RNA, free amino acids and rate of protein biosynthesis. However, adult animals that are fed this diet after exposure to γ-radiation are still able to mobilize their protein reserve and increase the various mechanisms for protein biosynthesis of their livers. If the protein reserve has been previously reduced, then the animals have a decreased ability to survive the effects of radiation (7). This substantiates the conclusion that optimal filling of this protein reserve is necessary for maximal response to stress conditions.

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Mechanism of the Cholesterol-depressing Effect of Pectin in the Cholesterol-fed Rat [1]

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ABSTRACT The mechanisms by which dietary pectin lowers plasma and liver cholesterol levels in cholesterol-fed rats were studied. Pectin feeding increased fecal bile acid excretion in cholesterol-fed rats. In vitro studies with inverted intestinal sacs demonstrated that pectin decreased taurocholic acid transport by approximately 50%. Rats responded to dietary pectin and cholestyramine, a known inhibitor of bile acid absorption, similarly. Cholesterol-4-^{14}C absorption was somewhat depressed by dietary pectin as evidenced by fecal radioactive cholesterol excretion and deposition of cholesterol-4-^{14}C in liver. The effect of pectin on plasma and liver cholesterol was not altered by dietary succinylsulfathiazole. These data are interpreted to suggest that the hypocholesterolemic effects of pectin are not mediated by an alteration of the intestinal microflora. The results of this study indicate that pectin lowers plasma and liver cholesterol levels in cholesterol-fed rats primarily by inhibiting bile acid absorption and also by reducing cholesterol absorption.

A number of publications have demonstrated a relationship between the incidence of atherosclerosis and serum cholesterol (1). These observations have naturally led to the study of factors influencing cholesterol metabolism and serum cholesterol levels. The control of serum cholesterol levels by dietary means has received particular attention (2). Dietary pectin has been reported to depress serum and liver cholesterol levels of cholesterol-fed rats (3) and chickens [2] and to reduce serum cholesterol levels in man (4).

The mechanism by which dietary pectin exerts its hypocholesterolemic effect has not been elucidated. Apparently, pectin exerts its effect in part by depressing cholesterol absorption (5). Other possibilities are 1) that the effects of dietary pectin might be mediated by an alteration of the intestinal microflora, or 2) that pectin may decrease the enterohepatic circulation of bile acids by interfering with bile acid absorption. These possible mechanisms have been studied in the rat and data are presented which suggest that the hypocholesterolemic effect of pectin is mediated primarily by an inhibition of bile acid absorption and also by a depression of cholesterol absorption.

MATERIALS AND METHODS

Male rats of the Holtzman strain were used for all studies. The animals were housed in stainless steel cages having raised wire floors; the room temperature and humidity were controlled (21° and 50% relative humidity). Diets were supplied ad libitum except in one experiment in which 2 diets were fed alternately; in this experiment animals were allowed access to food from 4:00 PM until 8:00 AM only. Water was supplied ad libitum. Body weight and food consumption were determined weekly. The experimental diets were fed for 3 or 4 weeks as indicated in the tables of results.

The basal diet had the following composition: (in g/100 g) casein (vitamin free), 18; L-cystine, 0.3; non-nutritive fiber, 3; salt mix (USP XIV), 4; corn oil, 5; vitamin mixture,[3] 0.4; choline Cl, 0.3; cholesterol, 1; glucose to 100. All additions to the basal diet, as indicated in the table of results, were made at the expense of glucose.

At the termination of the experiments, blood was obtained by cardiac puncture, while the animals were under light ether anesthesia, using heparin as an anticoagulant. The rats were then killed by exposure to chloroform and the livers were excised, blotted to remove excess blood, weighed and stored at − 20° until analysis

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[1] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

[2] Fisher, H., P. Griminger and W. Siller 1965 Retardation of cholesterol-induced atherosclerosis by pectin. Federation Proc., 24: 263 (abstract).

[3] For composition see Leveille et al. (6).

was performed. Plasma and liver lipids were determined as previously described (7, 8).

Fecal lipids were determined on pooled 3-day samples per rat at the termination of the study (table 1). The feces were dried at 60° under reduced pressure, weighed and ground to a fine powder. An aliquot of the dried, ground feces was extracted with chloroform:methanol (2:1, v/v) and 3-β-hydroxy sterols were determined by the same procedure employed for plasma and liver cholesterol. A second aliquot of the dried feces was analyzed for di- and trihydroxy bile acids by the method of Mosbach et al. (9).

Cholesterol absorption was studied with cholesterol-4-^{14}C. Rats fed the experimental diets for 3 weeks were given by stomach tube 2.8 μc of cholesterol-4-^{14}C dissolved in 1 g of corn oil. The feces were collected for 24 hours, saponified and cholesterol was isolated as the digitonide (10) and the radioactivity determined in a Nuclear-Chicago Corporation Model 722 ambient temperature liquid scintillation spectrometer.

In vitro bile acid absorption was studied by the inverted sac technique of Wilson and Wiseman (11). Rats weighing 400 g and fed commercial laboratory chow [4] from weaning were used. The animals were killed by exposure to ether; the lower one-fourth of the small intestine was removed and placed in cold saline containing 0.1% glucose; the intestine was washed with the same saline solution and inverted. Sacs of approximately 3 cm were prepared and filled with Krebs-Ringer bicarbonate buffer containing 100 μg/ml of taurocholic acid. The sacs were incubated for 90 minutes in beakers containing 10 ml of the same buffer containing taurocholic acid, with or without the addition of 0.18% pectin, at 38° in a Dubnoff metabolic shaker at a shaking rate of 90 oscillations/minute. The gas phase was 95% O_2 and 5% CO_2. At the end of the incubation period, the taurocholic acid content of the incubation medium (mucosal) and the medium within the sacs (serosal) was determined by the modified Pettenkofer method of Irwin et al. (12).

The data were statistically evaluated by means of the *t* test.

RESULTS

The effects of dietary pectin on body weight gain, plasma cholesterol, liver lipids, and fecal sterol and bile acid excretion in cholesterol-fed rats are shown in table 1. Pectin did not alter body weight gain. Plasma cholesterol and liver cholesterol levels were significantly reduced by dietary pectin. Fecal sterol excretion was not altered by pectin feeding; bile acid excretion was increased by 31.8% in rats fed pectin; however, this difference did not attain statistical significance at the 5% level.

To further investigate the possibility that pectin lowered cholesterol levels in cholesterol-fed rats by impairing bile acid absorption, the effects of pectin were compared with those of cholestyramine, a resin known to bind bile acids in the intestine

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

TABLE 1

Body weight gain, plasma cholesterol, liver fat and cholesterol and the fecal excretion of sterols and bile acids in rats fed a cholesterol-supplemented diet with or without added pectin

	Additions to diet		*P* values [1]
	Cholesterol (1%)	Cholesterol (1%) + pectin (5%)	
28-day body wt gain, g	181 ±5 [2]	173 ±11	ns
Plasma cholesterol, mg/100 ml	128 ±3	116 ± 5	< 0.05
Liver fat, % [3]	7.4±0.3	6.6± 0.3	ns
Liver cholesterol, mg/g [3]	10.3±0.5	7.5± 0.3	< 0.01
Fecal sterols, mg/day [4]	142 ±5	141 ± 8	ns
Fecal bile acids, mg/day [4]	17.9±2.4	23.6± 3.3	ns

[1] Probability of differences being significant; ns = not significant ($P > 0.05$).
[2] Mean ± SE of mean for 10 rats.
[3] Liver lipid values expressed on a wet-weight basis.
[4] Values are means for 5 animals.

TABLE 2

Body weight, liver fat and cholesterol, plasma total lipids and cholesterol of rats fed pectin or cholestyramine with supplemental cholesterol [1]

Dietary treatment	Body wt	Liver [2]		Plasma	
		Fat	Cholesterol	Total lipids	Cholesterol
	g	*%*	*mg/g*	*mg/100 ml*	*mg/100 ml*
1% Cholesterol	272 ± 14 [3]	7.9 ± 0.3	10.3 ± 0.5	910 ± 114	128 ± 14
1% Cholesterol + 5% pectin	269 ± 9	7.1 ± 0.4	7.2 ± 1.1 [4]	719 ± 81	91 ± 4 [4]
1% Cholesterol + 1% cholestyramine	258 ± 19	5.8 ± 0.2 [4]	4.0 ± 0.2 [4]	736 ± 57	86 ± 4 [4]
Diets alternated daily:					
3% Cholesterol and cholesterol-free	251 ± 12	6.5 ± 0.3	7.8 ± 0.8	723 ± 34	90 ± 3
3% Cholesterol and 5% pectin	235 ± 22	5.4 ± 0.3 [4]	4.2 ± 0.6 [4]	743 ± 38	101 ± 12
3% Cholesterol and 1% cholestyramine	260 ± 6	6.0 ± 0.2	5.0 ± 0.2 [4]	776 ± 32	110 ± 4 [4]

[1] Rats weighing approximately 150 g were fed the diets indicated for 3 weeks.
[2] Liver values are expressed on a wet-weight basis.
[3] Mean for 5 rats ± SE of mean.
[4] Values significantly different ($P < 0.05$) from group fed 1% cholesterol or fed alternately 3% cholesterol and the cholesterol-free diet.

TABLE 3

Effect of pectin on the in vitro absorption of taurochloric acid by sacs of inverted rat intestine [1]

Addition to mucosal fluid	Final serosal conc	Final serosal conc / Final mucosal conc
	μg/ml	
None	297 ± 46 [2]	3.28 ± 0.56
Pectin, 0.18%	158 ± 11	1.52 ± 0.08
P [3]	< 0.05	< 0.05

[1] Intestinal sacs (~ 3 cm) were prepared from the distal one-fourth of the intestine of 400-g rats. Serosal fluid: Krebs-Ringer bicarbonate containing 100 μg taurocholic acid; mucosal fluid: 10 ml bicarbonate buffer ± 0.18% pectin N.F. Preparation incubated 90 minutes at 38° under 95% O_2 and 5% CO_2.
[2] Mean for 4 experiments ± SE of mean.
[3] Probability of the difference being significant.

and thereby prevent their absorption (14). Rats were fed cholesterol-supplemented diets containing either pectin (5%) or cholestyramine (1%) for 3 weeks or were fed on alternate days a diet containing 3% cholesterol and one containing either 5% pectin or 1% cholestyramine.[5] The results of this experiment are shown in table 2. The effects of pectin and cholestyramine were similar whether fed together with cholesterol or separately. These data further suggest that pectin may act by impairing bile acid absorption.

The influence of pectin on bile acid absorption was studied in vitro with inverted intestinal sacs. Sacs from the distal one-fourth of the small intestine were used since it had been demonstrated that bile acid absorption is limited to this area (13). Sacs incubated with or without pectin added to the incubation medium concentrated taurocholate; however, pectin reduced the amount transported by approximately 50% as judged by the serosal-to-mucosal ratio or by the final serosal concentration (table 3). These data support the concept that the hypocholesterolemic effect of dietary pectin in the rat is mediated by an impairment of the enterohepatic circulation of bile acids.

The possible influence of an altered intestinal microflora in pectin-fed rats was studied by feeding pectin to animals receiving 1% dietary cholesterol with or without 1% succinylsulfathiazole. These data are presented in table 4. Succinylsulfathiazole significantly increased plasma cholesterol and liver fat and cholesterol levels ($P < 0.05$). Pectin was as effective in lowering plasma cholesterol and liver lipids in the presence or absence of dietary succinylsulfathiazole, suggesting that its hypocholesterolemic effect is not mediated by an alteration of the intestinal flora.

[5] The cholestyramine used in these studies was generously supplied by Dr. H. D. Brown, Research Laboratories, Merck, Sharp and Dohme, Rahway, New Jersey.

TABLE 4

Plasma cholesterol, liver fat and cholesterol levels of rats fed pectin with or without supplemental succinylsulfathiazole [1]

Dietary variables [2]	Plasma cholesterol	Liver [3]	
		Fat	Cholesterol
	mg/100 mg	%	*mg/g*
Cholesterol	86 ± 5 [4]	5.2 ± 0.4	10.2 ± 1.1
Cholesterol + pectin	77 ± 3	4.2 ± 0.1	4.8 ± 0.1
P [5]	ns [6]	< 0.05	< 0.01
Cholesterol + sulfa	136 ± 8	7.8 ± 0.4	20.7 ± 1.5
Cholesterol + sulfa + pectin	98 ± 3	5.5 ± 0.3	8.5 ± 0.9
P [5]	< 0.01	< 0.01	< 0.01

[1] Rats weighing approximately 50 g were fed the experimental diets for 4 weeks.
[2] Cholesterol 1%; pectin N.F. 5%; Sulfa = succinylsulfathiazole 1%.
[3] Liver values are expressed on a wet weight basis.
[4] Mean for 10 rats ± SE of mean.
[5] Probability of the difference being significant; ns = not significant.
[6] Indicates not significant.

TABLE 5

Liver fat and cholesterol, cholesterol-4-^{14}C deposition in liver and fecal excretion in rats fed cholesterol-supplemented diets with or without added pectin [1]

	Additions to diet		P value [2]
	Cholesterol (1%)	Cholesterol (1%) + pectin (5%)	
Liver fat, % [3]	6.8 ± 0.4 [4]	4.4 ± 0.2 [4]	< 0.001
Liver cholesterol, mg/g [3]	9.4 ± 0.4	6.4 ± 0.2	< 0.001
Cholesterol-^{14}C [5] DPM × 10^3/g liver	51.0 ± 5.8	37.4 ± 0.2	ns [6]
Fecal excretion, % of administered dose	6.3 ± 2.1	13.1 ± 3.4	ns

[1] Rats weighing approximately 250 g were fed the experimental diets for 3 weeks.
[2] Probability of difference being significant; ns = not significant.
[3] Liver values expressed on a wet-weight basis.
[4] Mean for 4 rats ± SE of mean.
[5] 2.8 μc of cholesterol-4-^{14}C in 1 g corn oil were fed by stomach tube; fecal excretion represents radioactivity excreted in 24-hour period following administration.
[6] Indicates not significant.

In table 5 are shown the results of an experiment designed to study the influence of dietary pectin on cholesterol absorption. Again, pectin lowered liver lipids in cholesterol-fed rats. Rats fed pectin excreted over twice as much of the administered radioactive cholesterol as did control animals; however, probably because of the small numbers this difference did not attain statistical significance. However, the difference in excretion of radioactive cholesterol and the lower amount of cholesterol-4-^{14}C in the liver of pectin-fed animals suggests that pectin does in fact depress cholesterol absorption.

DISCUSSION

The series of experiments reported was designed to evaluate the mechanisms by which dietary pectin depresses liver cholesterol levels in the cholesterol-fed rat. Since pectin is generally considered not to be digested, it follows that its effects would probably be limited to the gastrointestinal tract. The most likely mechanisms appeared to be related to 1) a reduction in cholesterol absorption, 2) a depression of bile acid absorption or recirculation, and 3) an alteration in the intestinal microflora. The possible involvement of these 3 mechanisms were therefore evaluated.

The most important effect of pectin appears to be its influence on bile acid absorption. Pectin clearly impairs bile acid absorption in vitro. Such experiments do not prove that the same effect would be found in vivo; however, when considered together with the greater bile acid excretion observed in pectin and cholesterol-fed rats (table 1), the similarity in response of the cholesterol-fed rat to pectin and a known inhibitor of bile acid absorption, cholestyramine (table 2), and the general agreement between in vitro and in vivo studies of bile acid absorption (13, 15), the data strongly suggest that pectin does in fact impair bile acid absorption.

The decrease in cholesterol absorption observed in this study as a consequence of pectin feeding is in agreement with the reports of Lin et al. (5) and of Hyun et al. (16). However, the impairment in cholesterol absorption is not evidenced when sterol excretion is determined (table 1). This observation is in agreement with the results of Wells and Ershoff (3) who were unable to demonstrate a difference in cholesterol excretion of rats fed a cholesterol-supplemented diet with or without pectin. It appears therefore that the impaired cholesterol absorption induced by dietary pectin is only partially responsible for its hypocholesterolemic effect. This conclusion is also supported by the study in which cholesterol and pectin were fed separately on alternate days (table 2). These results are in agreement with those of Wells and Ershoff (3). The observation that pectin effectively lowers liver cholesterol when fed separately from cholesterol suggests that impairment of cholesterol absorption is relatively unimportant to the overall effect of dietary pectin.

The alterations in the intestinal microflora resulting from pectin feeding do not appear to contribute to the cholesterol-lowering effect of this complex carbohydrate. The lack of effect of succinylsulfthiazole on the pectin response observed in this study is in agreement with the observations of Wells and Ershoff (3) and is in accord with the conclusion that alterations of the intestinal microflora do not influence the cholesterolemic effect of dietary pectin in the rat.

Fisher et al. (17) have recently presented evidence that in the chicken, pectin interferes with absorption of nutrients by increasing passage of food through the digestive tract. This appears to be unlikely in the rat in view of the consistent lack of effect of dietary pectin on body weight (tables 1 and 2).

The data presented suggest that dietary pectin reduces plasma and liver cholesterol levels in the cholesterol-fed rat primarily by decreasing the absorption of bile acids and secondarily that of cholesterol.

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Distribution of Lipids in Rats Fed 1,3-Butanediol

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ABSTRACT Quantitative thin-layer chromatography was used to measure the level of individual lipid components of liver, plasma and adipose tissue in rats fed 1,3-butanediol (BD) as a replacement of "natural" carbohydrate. The addition of 20% BD to diets containing either 10 or 30% fat resulted in a slight increase in liver neutral lipids, specifically, triglycerides and cholesterol esters. Phospholipid and cholesterol levels were not affected by an increase in dietary fat or BD. Plasma levels of non-esterified fatty acids, triglycerides and cholesterol esters were also higher as a result of increases in dietary fat or supplementation with BD. Addition of BD to either the 10 or 30% fat diet resulted in a highly significant decrease in epididymal fat.

Ever since 1,3-butanediol (BD) was shown to be a potential source of dietary energy (1–3), efforts have been directed toward elucidating its metabolic effects. Previous work in these laboratories has shown that the feeding of BD to rats results in changes in tissue total lipid concentrations (4). Whether the BD effect on lipid metabolism is general, affecting all classes of tissue lipids, or whether it interferes with a particular lipid class is not known. The present paper reports the results of an investigation on the effect of prolonged feeding of BD as a replacement of "natural" carbohydrate on the individual lipid components of liver, plasma and adipose tissue of laboratory rats.

EXPERIMENTAL METHODS

Animals and diets. Male rats of the Sprague-Dawley strain were housed in individual cages in a thermostatically controlled room at 25 ± 1°. Thirty-two rats with a body weight of 90 to 100 g were divided into 4 equally weighted groups of 8 animals per group and fed the following 4 diets: 1) 10% fat, 2) 10% fat and 20% BD, 3) 30% fat and 4) 30% fat and 20% BD. The composition of these diets was presented previously (5). The diets were fed ad libitum for 5 weeks. Food consumption and rat weights were measured bi-weekly. The animals were decapitated after an 18-hour fast.

Tissue extractions. Liver was immediately excised, weighed and a 1 to 2 g sample removed and homogenized in chloroform-methanol (2/1, v/v). The homogenized samples from the individual rats in each group were pooled and the lipids extracted and measured as described by Therriault and Poe (5). Both epididymal fat pads were quantitatively excised from each animal and weighed. Lipid extraction and determination was carried out on the combined fat pads of each rat.

A 1-ml aliquot of plasma from each rat in a group was pooled. The pooled samples were acidified slightly with dilute HCl and extracted with chloroform-methanol (2/1). The aqueous layer was re-extracted an additional 3 times with chloroform-methanol (2/1). The chloroform layers were combined, evaporated to dryness in vacuo under nitrogen and taken up in a small amount of chloroform-methanol (2/1).

Lipid fractionation. Neutral lipids and free fatty acids were separated from phospholipids by chromatography on columns prepared with pre-washed silicic acid [4] (100-mesh). Silicic acid was thoroughly washed with chloroform-methanol (2/1), followed by methanol. The washed silicic acid was activated at 105° for 24 hours.

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A column (1.4 × 10 cm) was prepared by packing the silicic acid as a slurry in chloroform under a slight nitrogen pressure. The column was then washed with chloroform. The lipid sample was applied in 2 ml of chloroform. Neutral lipids and free fatty acids were eluted with 40 ml of chloroform. Phospholipids were eluted with 60 ml of methanol. Thin-layer chromatography of the fractions showed that the chloroform eluate was free of phospholipid and the phospholipid fraction was free of neutral lipids and free fatty acids. Recovery of neutral lipids and free fatty acids was 98 to 102%. The phospholipid was calculated by the difference between total lipid and neutral lipid.

Quantitative thin-layer chromatography of neutral lipids and free fatty acids. Quantitative determination of the lipids contained in the chloroform eluate was carried out by thin-layer chromatography according to the method described by Louis-Ferdinand et al. (6). A hexane-ethyl ether-acetic acid (180/30/2) solvent system was used for the determination of cholesterol esters and triglycerides, and petroleum ether-ethyl ether-acetic acid (30/70/1) was used to determine cholesterol and non-esterified fatty acids. Highly purified lipid standards, containing cholesterol, cholesterol oleate, oleic acid, methyl oleate and triolein, obtained from Hormel Institute were applied to the chromatoplate alongside each sample. In preliminary studies, the total lipid obtained by thin-layer chromatography agreed within 10% with gravimetric determination of the lipid after separation by silicic acid column chromatography. A Photovolt Densitometer modified for measurement of chromatoplates was used to measure spot densities. Determinations were carried out in quadruplicate and the maximal difference in replicate samples was less than 8%.

RESULTS

The 5-week food consumption values shown in table 1 confirm earlier observations (3) that young animals fed diets containing BD failed to gain as much body weight as control rats, due to reduction in food intake. Weight gains were significantly lower in rats fed diets containing 20% BD than those of the controls.

The epididymal fat pad weight and total lipids are presented in table 2. Both tissue weight and lipid content increased as dietary fat level increased. This is in agreement with well-established effects of fat intake on lipid deposition (7). However, the addition of BD to either diet resulted in a highly significant decrease in the

TABLE 1

Food intake and weight gain of rats fed experimental diets [1,2]

Fat content	BD [3]	Food intake/rat [4]	Wt gain/rat
%		*g*	*g*
10	−	559.6	193.5 ± 15.8 [5]
	+	459.8	145.9 [6] ± 17.8
30	−	448.6	178.1 ± 20.8
	+	403.0	143.9 [6] ± 13.4

[1] Figures represent cumulative results over 34-day period.
[2] Eight rats/group.
[3] 1,3-Butanediol.
[4] Grams dry weight of food.
[5] SD.
[6] Significant differences at $P < 0.001$ level. Student's *t* test.

TABLE 2

Weight and lipid content of epididymal fat tissue from rats maintained with experimental diets [1]

Fat in diet	BD [2]	Wet wt tissue	Total lipids	Tissue	Lipids
%		*g*	*g*	*g/100 g body wt*	*g/100 g body wt*
10	−	2.98 ± 0.23	2.59 ± 0.24 [3]	1.09	0.94
	+	2.14 ± 0.29 [4]	1.84 ± 0.25 [4]	0.90	0.77
30	−	3.92 ± 0.85	3.52 ± 0.78	1.41	1.27
	+	1.97 ± 0.21 [4]	1.70 ± 0.19 [4]	0.86	0.74

[1] Five rats/group.
[2] 1,3-Butanediol.
[3] SD.
[4] Significant difference at $P < 0.001$ level. Student's *t* test.

TABLE 3

Liver weight and liver lipid composition of rats maintained with experimental diets [1]

Fat in diet	BD [2]	Body wt	Liver wt	Lipid in liver
%		g	g	%
10	−	274.6 ± 10.6 [3]	7.9 ± 0.4	5.44
	+	237.4 ± 4.5 [4]	7.4 ± 0.4	6.06
30	−	276.8 ± 8.4	8.3 ± 0.7	6.98
	+	229.6 ± 4.4 [4]	8.0 ± 0.4	7.31

[1] Five rats/group.
[2] 1,3-Butanediol.
[3] SD.
[4] Significant differences at $P < 0.001$ level.

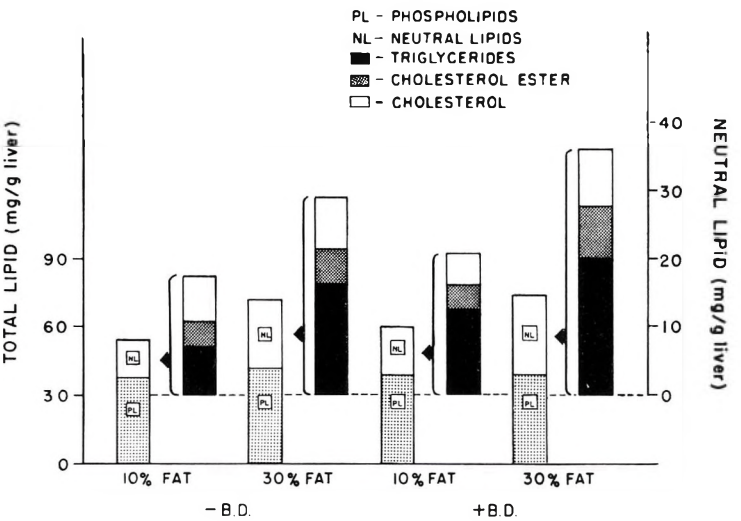

Fig. 1 Liver lipid composition of rats fed 10% or 30% fat with or without 1,3-butanediol.

tissue weight as well as the total lipid content of the epididymal fat pad.

Liver total lipid concentrations as well as total liver lipid mass were also increased when dietary fat levels were changed from 10% to 30% as shown in table 3. This is in agreement with previous reports (8). Addition of BD to these diets did not result in any striking change in liver total lipid concentration or mass. However, there was a change in lipid composition of liver (fig. 1). An increase in neutral lipid concentration occurred in response to changes of dietary fat levels or the addition of BD. This is evident specifically in the triglyceride fraction and to a lesser extent in the cholesterol ester fraction. The phospholipids and cholesterol which account for the greatest portion of the liver total lipids were not affected either by the presence of BD or the dietary fat content.

As shown in figure 2, both increase in dietary fat and the presence of BD resulted in increased plasma unesterified fatty acids (FFA) triglycerides and cholesterol ester. As in the liver, the free cholesterol content of plasma did not respond to changes in dietary fat levels or the addition of BD.

DISCUSSION

Plasma FFA represents the form in which fatty acids are mobilized from the fat depots and transported to other tissues (9). In the fasted state, fatty acid metabolism is geared to provide substrate for oxidation by the tissues and there is a

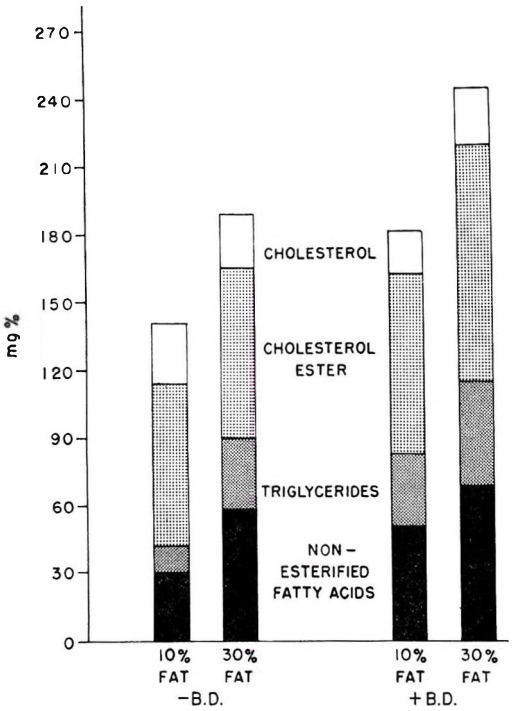

Fig. 2 Plasma neutral lipid and free fatty acid composition of rats fed 10% or 30% fat with or without 1,3-butanediol.

large net flux of FFA from adipose tissue to other tissues. In the results reported here, the elevated plasma FFA level in both groups receiving BD indicates that a greater mobilization of lipid from adipose tissue occurred in these animals. This is further evident from their smaller fat pads.

Studies by Feigelson et al. (10) have shown that when the serum FFA concentration is maintained at a high level, there is a rapid and marked increase in the triglyceride concentration of liver. Eaton and Steinberg (11) have had similar results in myocardial tissue and postulated that the extraction of FFA from plasma by muscle tissue may be primarily regulated by the levels of circulating FFA. It is conceivable that the slightly higher triglyceride and cholesterol ester levels in liver of rats fed BD may be the result of a greater influx of FFA from adipose tissue. The higher plasma triglyceride levels in rats fed BD indicate that there is no impairment in the ability to transport low density lipoprotein from the liver to plasma.

No information is available as to the effect of BD on fatty acid synthesis or utilization in the rat. Further investigation should be undertaken to see whether BD affects these parameters of lipid metabolism.

ACKNOWLEDGMENT

The authors acknowledge the technical assistance of Stephen Swift.

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Utilization of Calories and Nitrogen by Rats Fed Diets Containing Purified Casein versus a Mixture of Amino Acids Simulating Casein [1]

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ABSTRACT The effects were studied of varying nitrogen and energy intake levels on calorie and nitrogen storage in rats of 2 age groups. Male rats 28 or 100 days of age were fed for 4 and 6 weeks, respectively, diets containing either casein or an amino acid mixture simulating casein, at 2 levels of nitrogen and 2 levels of calorie intake. For the young rats, at the lower calorie intake and at both levels of nitrogen intake, there were no significant differences in nitrogen gains due to nitrogen source. At the higher calorie intake, and at both levels of nitrogen intake, nitrogen gains were higher for rats receiving casein than for those receiving amino acids. For the adult rats, at the lower nitrogen intake there were no significant differences in nitrogen gains (or losses) due to nitrogen source, regardless of the calorie intake. At the higher nitrogen intake and at both levels of calorie intake, animals receiving casein gained more (or lost less) nitrogen than rats receiving amino acids. It is postulated that a difference in the most limiting factor (whether energy or nitrogen) accounted for the difference in the results between the 2 age groups. For both the young and the adult rats, calories stored were unaffected by substituting an amino acid mixture for casein.

There has been a lack of agreement in research reports on the nutritional value of intact casein as compared with a mixture of amino acids simulating casein. Rose et al. (1) reported that human adults were able to maintain nitrogen balance at a lower level of energy intake with casein diets than with diets containing acid-hydrolyzed casein or a mixture of purified amino acids simulating casein. Metta et al. (2) reported that replacing dietary casein by a mixture of the corresponding amino acids or a casein hydrolysate did not increase the energy requirement for maintenance of nitrogen balance of male and female rats over 6 months of age. They attribute the variance of their results from those of Rose et al. (1) to a species difference. The purpose of this paper is to report the effects of varying nitrogen and energy intake levels on calorie and nitrogen balance in rats of 2 age groups fed casein diets or diets containing a mixture of L-amino acids simulating casein.

EXPERIMENTAL

Animals and diets. All animals were specific-pathogen-free male rats.[2] Weanling and 100-day-old rats were housed individually and fed a stock diet [3] for one week before being fed their particular experimental regimen. A control group of 12 rats was killed for each age to determine initial carcass content of calories and nitrogen and to furnish data for correcting initial weight of the experimental animals to the ingesta-free basis. Initial live weight was multiplied by 90.2 and 94.7% for the young and adult experimental rats, respectively. Contents of the gastrointestinal tract were removed and the carcasses homogenized in a Waring Blendor before analysis (3).

The animals were given weighed amounts of food daily and body weights were recorded weekly. Scattered food was carefully recovered and weighed. Feces were collected daily from each animal and frozen until the end of the feeding trial,

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[1] Preliminary results of the investigation were reported to the American Institute of Nutrition at the 49th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1965.

[2] Lew strain from Microbiological Associates, Bethesda, Maryland.

[3] D & G Research Animal Laboratory Diet, Price-Wilhoite Company, Frederick, Maryland.

then dried and ground. Feces from each rat were analyzed individually for nitrogen; calorie determinations were made on feces samples pooled for each group. The diets were analyzed for nitrogen and calories. The Kjeldahl method was used for all nitrogen analyses and calorie content was measured with a Parr Bomb Adiabatic Calorimeter.

The diets were fed at 2 levels of calorie intake and 2 levels of nitrogen intake, with either casein or an L-amino acid mixture simulating casein as the nitrogen source. For the young rats, diets contained either 2 or 3% nitrogen, and, for the adult rats, either 1 or 2% nitrogen (table 1). Corresponding diets were formulated with increases in protein or amino acids, vitamins, salts and roughage so that, when calorie intake was reduced approximately one-third, intakes of those nutrients were the same as the intakes at the higher calorie level. Thus, there were 8 diets for each age group. Fat and carbohydrate were reduced proportionally so that ratios of fat to carbohydrate, and of corn oil to tallow were the same for each calorie level. The calorie concentration of the casein diets was somewhat higher than that of the amino acid diets due to the higher calorie content of casein. However, the diets were formulated and the amounts fed adjusted so that intakes of nitrogen and calories were similar (table 2).

The 1% nitrogen diets contained, as the nitrogen source, either 6.82% casein or (in %) alanine, 0.187; arginine·HCl, 0.285; asparagine·H_2O, 0.492; cystine, 0.20; glutamic acid, 1.484; glycine, 0.138; histidine · HCl · H_2O, 0.226; isoleucine, 0.364; leucine, 0.560; lysine · HCl, 0.649; methionine, 0.187; phenylalanine, 0.315; proline, 0.777; serine, 0.433; threonine, 0.265; tyrosine, 0.334; tryptophan, 0.079; and valine, 0.452. In the 2 and 3% nitrogen diets the amounts of casein or amino acids were increased proportionally.

The casein [4] was assayed microbiologically using the methods of Horn et al. (5) for arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine, the method of Warren et al. (6) for alanine, of Horn and Blum (7) for cystine, and unpublished methods of the latter authors for all other amino acids. The amino acids were assayed for purity by the same methods. All amino

[4] A.N.R.C. reference protein, Sheffield Chemical Company, Norwich, Connecticut.

TABLE 1

Composition of diets fed at the higher calorie level to rats of 2 age groups

	Nitrogen source					
	Casein			Amino acid mix		
	1% N	2% N	3% N	1% N	2% N	3% N
Casein	6.82	13.63	20.45	—	—	—
Amino acid mix	—	—	—	7.25	14.25	21.02
Diammonium citrate	—	—	—	0.65	1.27	1.95
Sodium bicarbonate	—	—	—	0.50	0.99	1.45
Cornstarch	58.13	51.32	44.50	57.15	49.60	42.28
Corn oil [1]	5.00	5.00	5.00	4.91	4.83	4.75
Beef tallow	15.00	15.00	15.00	14.75	14.50	14.25
Vitamin A and D conc [2]	0.05	0.05	0.05	0.05	0.05	0.05
Salt mix [3]	4.00	4.00	4.00	3.93	3.87	3.80
Cellulose [4]	10.00	10.00	10.00	9.83	9.67	9.50
Vitamin mix [5]	1.00	1.00	1.00	0.98	0.97	0.95
Total	100.00	100.00	100.00	100.00	100.00	100.00
Gross calories, kcal/g	4.76	4.92	5.09	4.70	4.76	4.78

[1] Mazola, Corn Products Company, Argo, Illinois.
[2] Percomorph Oil, Mead Johnson Company, Evansville, Indiana.
[3] Jones and Foster (4).
[4] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[5] The following vitamins were added per kilogram of ration: thiamine·HCl, 5 mg; pyridoxine·HCl, 5 mg; niacin, 5 mg; riboflavin, 10 mg; Ca D-pantothenate, 25 mg; p-aminobenzoic acid, 300 mg; α-tocopheryl acetate, 25 mg; 2-methyl-1,4-naphthoquinone, 2 mg; folic acid, 2 mg; biotin, 100 μg; vitamin B_{12}, 30 μg; choline chloride, 2 g; and inositol, 1 g.

TABLE 2

Weight gains, nitrogen and calorie intakes, and nitrogen and calorie storage of young and adult rats fed at 2 levels of nitrogen, either as amino acids or as casein, at 2 levels of calorie intake

N source [1]	Intakes		Wt gain [2]	Nitrogen stored	Calories stored	Non-protein calories stored
	N	Calories				
	g	*kcal*	*g*	*g*	*kcal*	*kcal*
			Young rats [3]			
AA	5.46	1301	87 ± 1.9 [4]	2.88 ± 0.07 **	203 ± 9.6	101 ± 8.5
C	5.56	1351	96 ± 0.8 **	3.34 ± 0.06 **	220 ± 5.9	102 ± 7.2
AA	8.09	1333	106 ± 1.8 **	3.72 ± 0.08 **	236 ± 5.9	105 ± 6.3
C	8.08	1366	113 ± 1.4 **	4.11 ± 0.04 **	236 ± 4.5	91 ± 5.1
AA	5.77	952	52 ± 1.4	2.18 ± 0.06	98 ± 4.8	21 ± 5.3
C	5.70	940	55 ± 2.0	2.30 ± 0.09	107 ± 3.8	26 ± 2.9
AA	8.35	961	55 ± 1.8	2.30 ± 0.06	104 ± 4.1	22 ± 4.6
C	8.06	928	57 ± 1.3	2.38 ± 0.07	101 ± 3.7	18 ± 3.6
			Adult rats [5]			
AA	7.06	3132	36 ± 3.1	0.09 ± 0.11	410 ± 22.1	407 ± 21.9
C	6.89	3037	34 ± 2.4	0.21 ± 0.12	367 ± 21.0	360 ± 21.0
AA	12.81	3114	52 ± 2.1 *	0.79 ± 0.15 *	415 ± 19.0	390 ± 18.8
C	12.95	3049	59 ± 2.4 *	1.28 ± 0.12 *	453 ± 20.4	408 ± 21.5
AA	7.13	2254	−27 ± 3.0	−0.66 ± 0.09	21 ± 14.1	44 ± 11.8
C	7.05	2269	−24 ± 3.5	−0.51 ± 0.10	27 ± 14.6	45 ± 13.6
AA	14.04	2311	−23 ± 2.7	−0.53 ± 0.15 *	40 ± 11.5	59 ± 11.3
C	13.46	2267	−17 ± 3.1	−0.04 ± 0.15 *	30 ± 15.9	31 ± 15.7

[1] C = casein; AA = amino acid mixture.
[2] Live weight gains. Nitrogen and calorie storage were calculated from ingesta-free carcass gains (see text). Average initial weights of the groups of young rats were 64 to 70 g, and of the adult rats 361 to 363 g.
[3] Totals for 4 weeks.
[4] SE of mean.
[5] Totals for 6 weeks.
* Designated values are significantly different ($P < 0.05$).
** Designated values are significantly different ($P < 0.01$).

acids except valine were found to be as pure as the standards.[5] Growth responses of the microorganisms were 10% below the response to the standard for the latter amino acid; accordingly, the amount of valine in the mixture was increased by 10%. Except for valine, the composition of the casein, in grams per gram of nitrogen is shown by the amounts of amino acids given in the preceding paragraph, when the appropriate corrections are made for H_2O and HCl.

In view of the report of a specific role of dietary asparagine (8) in the nutrition of the rat, asparagine, instead of aspartic acid, was included in the amino acid mixture. A small amount of diammonium citrate was added to make the diets isonitrogenous.

Study with young rats. Eight groups of weanling rats (12/group) were fed for 4 weeks the diets already described. The 3% nitrogen diets met the amino acid requirements for rats of this age as calculated from the data of Rama Rao et al. (9), whereas the 2% nitrogen diets were limiting in threonine. Either level of nitrogen should be adequate to meet the total protein and nonessential amino nitrogen requirements (10). Gross calorie intakes were approximately 34 or 48 kcal/day; nitrogen intakes, approximately 200 or 300 mg/day for the various groups. Animals fed the amino acid diets at the higher calorie intake in some cases did not consume all their food. Those whose calorie intake was not at least 92% of that of the animals receiving casein were eliminated. In so doing, the numbers for the group receiving 48 kcal and 200 mg N/day were reduced to ten and for the group fed 48 kcal and 300 mg N/day to eleven.

Study with adult rats. Eight groups of 100-day-old rats (12/group) were fed for 6 weeks diets already described. The

[5] U.S.P. reference standards where available; for the others, AP grade of the H. M. Chemical Company, Ltd., Santa Monica, California.

amount of food initially supplied resulted in weight loss; accordingly after the second week, food was increased each week by an amount that supplied 5 kcal/day. Average gross calorie intakes for the period were approximately 54 or 74 kcal/day; nitrogen intakes, approximately 170 or 315 mg/day for the various groups. One animal in the group which received 74 kcal and 315 mg N/day as casein was sick on the last day of the feeding period, and was eliminated from the group.

Non-fecal nitrogen loss was calculated by subtracting fecal nitrogen and nitrogen gain from nitrogen intake. This nitrogen loss, mainly urinary nitrogen, was multiplied by a factor of 6.29 kcal/g N as developed by Metta and Mitchell (11) with high protein diets in order to calculate urine calorie loss.

Both experiments are randomized complete block experiments with 12 blocks and 8 treatments. The 8 treatments lend themselves to a 2×2×2 factorial analysis for both nitrogen and calorie storage.

RESULTS AND DISCUSSION

Fecal nitrogen and calorie losses. Average fecal nitrogen values of the young rats (total for 28 days) were 0.32 to 0.37 g for the groups fed amino acids and 0.37 to 0.40 g for those fed casein. For the adult rats, average fecal nitrogen values (total for 42 days) were 0.87 to 1.06 g for the groups fed amino acids and 0.98 to 1.11 g for those fed casein. Average fecal calories for the groups of young rats varied from 175 to 214 kcal. Assuming that all ingested cellulose [6] was excreted, cellulose calories would account for 50 to 59% of the total. Average fecal calories for the groups of adult rats varied from 465 to 581 kcal. The cellulose calories would account for 43 to 55% of the total.

Weight gains and nitrogen storage. Young rats with the higher calorie intake and either level of nitrogen intake gained more weight and stored more nitrogen ($P < 0.01$) when the nitrogen was supplied as casein rather than as a mixture of purified amino acids (table 2). The significance of the differences in nitrogen storage was the same whether the data were expressed as total amount stored, or as a percentage of either gross or apparent digestible nitrogen intake; hence only data for total amount stored is presented. At the lower calorie intake there were no significant differences in weight gains or nitrogen storage between comparable groups.

Adult rats with the higher calorie and nitrogen intake gained more rapidly ($P < 0.05$) when the nitrogen was supplied as casein rather than as a mixture of purified amino acids. At the lower calorie and nitrogen intake, there were no significant differences in weight gains between those rats receiving casein and those fed amino acids.

At the higher level of nitrogen intake and at either level of calorie intake, adult rats fed casein stored more or lost less nitrogen ($P < 0.05$) than those fed a mixture of amino acids. At the lower level of nitrogen intake there were no significant differences in nitrogen storage between groups at the same calorie intake level.

Calorie storage. Calorie storage of both young and adult rats was unaffected by substituting an amino acid mixture for casein in the diet. This was true at both calorie and nitrogen levels fed and regardless of whether the calorie storage is expressed as kilocalories or as a percentage of gross, digestible or metabolizable calorie intake.

Analysis of variance. An analysis of variance (table 3) showed that, as would be expected, both calorie and nitrogen storage were altered in either age group of rats by varying the calorie or nitrogen intake ($P < 0.01$). There was, however, a significant energy level-nitrogen level (EN) interaction ($P < 0.01$) for nitrogen storage in both experiments. Table 2 shows that increasing nitrogen intake led to a larger increase in nitrogen storage at the high than at the low calorie intake in each age group. The EN interaction for calorie storage was significant ($P < 0.01$) for the young rats but not for the adult rats. This difference between the 2 age groups may be due to a difference in limiting factors (namely, energy or nitrogen) in the 2 experiments or to basic differences in the metabolism of the rat during growth and maturity. All groups of young rats stored calories and nitrogen; all groups of adult

[6] Cellu Flour, Chicago Dietetic Supply House, Chicago.

TABLE 3

Mean squares of analysis of variance for nitrogen and calorie storage

Source of variation	Young rats		Adult rats	
	Calorie storage	Nitrogen storage	Calorie storage	Nitrogen storage
	$kcal^2$	g^2	$kcal^2$	g^2
Blocks	396	0.0478	9,615	0.4195
Energy level (E)	353,322**	35.9415**	3,526,671**	25.7401**
Nitrogen level (N)	3,407**	4.9323**	20,644**	8.6456**
Nitrogen source (S)	817	1.6330**	14	2.4923**
EN	3,338**	2.9891**	8,200	2.2291**
ES	177	0.6700**	20	0.0001
NS	1,001	0.0183	7,420	0.8638*
ENS	29	0.0017	16,111*	0.0077
Error	327	0.0556	2,887	0.1548

** Significant ($P < 0.01$).
* Significant ($P < 0.05$).

rats stored calories but those receiving the lower calorie intake were in negative nitrogen balance and even the groups fed the higher calorie intake were barely in positive nitrogen balance. Extremely high nitrogen storage in 100-day-old rats would not be expected.

Support for a theory of different limiting factors in the 2 experiments is obtained by examining the energy level-nitrogen source (ES) and the nitrogen level-nitrogen source (NS) interactions on nitrogen storage. For the young rat the ES but not the NS interaction was significant ($P < 0.01$). Table 2 shows that there was higher nitrogen storage from casein than from the amino acid mixture at the high, but not the low, calorie intake level at both levels of nitrogen intake. For the adult rats the NS but not the ES interaction was significant ($P < 0.05$) indicating higher nitrogen storage from casein at the high, but not the low, nitrogen intake.

A significant energy level-nitrogen level-nitrogen source (ENS) interaction for calorie storage for the adult rats ($P < 0.05$) prompted a further examination of the EN, ES, NS interactions. The NS interaction was significant ($P < 0.05$) at the high but not at the low level of calorie intake. Fat storage may be a factor here. When the nitrogen intake was low and the calories were high, there was a trend toward higher storage of non-protein calories (fat) when the source of nitrogen was amino acids rather than casein.

At all calorie and nitrogen levels and for both age groups, rats receiving amino acid diets had greater non-fecal nitrogen losses ($P < 0.05$), via increased urinary excretion of unchanged amino acids or as a result of enzyme actions or both. (Since amino acid-fed rats lost slightly less nitrogen in the feces, there were not always significant differences in nitrogen storage, as shown in table 2.) Cohn et al. (12) suggest that there is a limit to the amount of dietary protein that an organism can utilize per unit of time for protein synthesis. When the limit is exceeded, "excess" nitrogen is excreted in the urine as enzymatic reactions adapt themselves to disposing of the non-utilized nitrogen. A difference in feeding habits was noted between the rats receiving amino acids and those receiving casein, particularly at the high calorie level; amino acid-fed rats tended to consume their diets more slowly. If feeding frequency was a primary factor in our results, therefore, rats receiving casein diets should have the highest non-fecal nitrogen losses. It is possible that the slower eating by rats fed the amino acids may be a compensatory mechanism designed to minimize what might otherwise be larger differences in non-fecal nitrogen loss.

Nasset and Ju (13) fed ^{14}C-labeled casein and observed that exogenous N was diluted 6- or 7-fold with endogenous N and the molar ratios of free amino acids in gut contents were markedly different from those noted in the ingested casein. Snook and Meyer (14) demonstrated that exogenous protein was the preferred substrate of intestinal proteolytic enzymes. A mixture of purified amino acids rather than

casein in the diet would be expected to alter the rate of secretion and breakdown of endogenous protein and the absorption of amino acids from the gut.

The significant energy level-nitrogen source (ES) interaction with young rats is in line with the earlier observations of Rose et al. (1) that higher calorie intakes are needed by subjects receiving amino acids than by those receiving casein. However, with adult rats there was no significant ES interaction, in line with the report of Metta et al. (2) that the energy requirement was not changed by replacing dietary protein with purified amino acids. As suggested earlier, differences in the limiting factors in the studies may be an explanation of differences in results. For purposes of comparison, intakes of the adult animals have been recalculated to intake/$kg^{3/4}$ (using, in the present study, mean body weight). Nitrogen intakes were 0.35 to 0.39 and 0.64 to 0.77 g/day/$kg^{3/4}$; metabolizable calorie intakes were 94 to 95 and 121 to 129 kcal/day/$kg^{3/4}$. Intakes of the male rats of the study of Metta et al. were 0.32 g nitrogen and 98 kcal/day/$kg^{3/4}$. Nitrogen intakes, then, were lower and calorie intakes similar, when related to metabolic body size, to those of the groups fed at the lowest levels of nitrogen and calories in the present study. For those groups there were no significant differences in nitrogen and calorie storage due to nitrogen source. Rose et al. (1) fed 0.40 to 0.44 g N/$kg^{3/4}$/day to human subjects, but a species difference in requirement may make this level of intake comparable to our high N intake groups and thus explain how Rose et al. (1) observed differences in nitrogen balance due to the nitrogen source, whereas Metta et al. (2) could find no such differences.

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Effect of Vitamin B_{12} and Folic Acid on the Metabolism of Formiminoglutamate, Formate, and Propionate in the Rat

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ABSTRACT The effects of deficiencies of vitamin B_{12} on excretion of formiminoglutamate (FIGLU), formate, and propionate were investigated. The excretion of FIGLU was increased in vitamin B_{12} deficiency and was higher with a 30% soy protein diet than with a 70% soy protein diet, although the vitamin B_{12} deficiency was more severe with the diet at higher protein level. Excretion of endogenous formate was increased in both folic acid and vitamin B_{12} deficiencies and roughly paralleled that of FIGLU. Excretion of injected ^{14}C-formate increased in folic acid deficiency but was less affected by vitamin B_{12} deficiency with a soy protein diet. The excretion of a test dose of ^{3}H-propionate was increased in vitamin B_{12} deficiency and developed more rapidly with the 70% than with the 30% soy protein diet. The feeding of either 2 or 4% calcium propionate produced a growth depression, but this was not appreciably diminished by vitamin B_{12}. Calcium propionate did not increase FIGLU excretion.

Deficiencies of both folic acid and vitamin B_{12} increase the excretion of formiminoglutamic acid (FIGLU) in rats (1–3) and chicks (4), although folic acid alone is known to be directly involved in the enzymatic breakdown of FIGLU to glutamic acid. The excretion of FIGLU has been used as a diagnostic test for clinical folic acid deficiency (5–7), but its usefulness is somewhat diminished because in some instances FIGLU excretion also occurs in the vitamin B_{12} deficiency of pernicious anemia (8). In order to throw light on the mechanism of action of vitamin B_{12} in affecting FIGLU metabolism, a study was made on the effect of various regimens which induce vitamin B_{12} deficiency. These include elevation of dietary protein level and the feeding of high levels of propionic acid (9, 10). Since the metabolism of propionate is known to be impaired in vitamin B_{12} deficiency in the rat (11) and the ruminant (12), the excretion of a test dose of propionate was studied as a possible parameter for assessing vitamin B_{12} deficiency. The effect of vitamin B_{12} deficiency on the metabolism of formate was also studied since the excretion of formate is increased in folic acid deficiency (2).

METHODS

Groups of 8 weanling male rats (2 rats/cage) were fed the experimental diets at 28 days of age. The composition of the basal diets is shown in table 1. A 30% soy protein diet and a 70% soy protein diet were used to produce the vitamin B_{12} deficiency. The 30% soy protein diet contained 0.27% methionine which is lower than the methionine requirement (in the presence of cystine) (13) of 0.4% for growth in the rat. No supplementary methionine was used because supplementary methionine is known to decrease FIGLU excretion (2–4) and it was deemed desirable to keep FIGLU excretion as high as possible in order to accentuate differences produced by dietary changes. The 30% soy protein diet was only marginally deficient with respect to amino acids as evidenced by the fact that growth with this diet, supplemented with folic acid and vitamin B_{12}, was 80% of that with a casein gelatin diet.

The basal diet was supplemented with 20 or 40 g/kg of calcium propionate with the objective of further increasing the need for vitamin B_{12}. The calcium content of all diets was kept constant by adjustment of the calcium carbonate used in the salt mixture to compensate for the calcium furnished by the calcium propionate. Since

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TABLE 1

Composition of basal diets

	30% Soy protein diet	70% Soy protein diet	20% Casein diet
	g/kg	*g/kg*	*g/kg*
Glucose monohydrate [1]	639	239	645
Soy protein [2]	300	700	—
Crude casein	—	—	200
Gelatin	—	—	80
Calcium carbonate	20	20	20
Salts [3]	20	20	20
Vitamin mixture [4]	10	10	10
Vitamins A, D, E, in corn oil [5]	10	10	10
Choline chloride	1	1	1
Cystine	—	—	4
Sulfasuxidine	—	—	10
Methionine content (calculated)	2.7 [6]	6.3	6.1
Histidine content (calculated)	7.0 [6]	16.5	4.5

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[3] Composition of salt mixture: (in grams) $CaCO_3$, 228; K_2HPO_4, 100; KH_2PO_4, 100; NaCl, 170; bone ash, 300; $MgSO_4$, 50; ferric citrate·$3H_2O$, 35; $MnSO_4 \cdot 4H_2O$, 10; $CuSO_4 \cdot 5H_2O$, 1; zinc acetate·$3H_2O$, 1; $Al_2(SO_4)_3$, 5; $CoCl_2$, 0.05; and $NiCO_3$, 0.02.
[4] The vitamin mixture furnished the following amounts/kg of diet: (in milligrams) riboflavin, 10; Ca pantothenate, 50; niacin, 50; pyridoxine, 10; thiamine, 10; and biotin, 0.2.
[5] Vitamins A, D, and E were supplied in the following amounts dissolved in 10 g of corn oil/kg of diet: vitamin A, 15,000 units; vitamin D, 2,000 units; and vitamin E, 70 units.
[6] Amino acid composition data supplied by manufacturer.

the soy protein was contaminated with appreciable amounts of folic acid, a casein diet low in folic acid was used for inducing folic acid deficiency for comparative purposes.

Metabolic tests were carried out in metabolism cages using 2 animals per group. The ^{14}C-formate excretion tests were made by giving about 1 μc of ^{14}C-formate intraperitoneally and measuring in a liquid scintillation counter the total radioactivity of the urine for the next 22-hour period using the solvent and phosphor mixture of Bray (14). The excretion of ^{3}H-propionate [3] was measured by administering 5 μc of propionate intraperitoneally and collecting the urine for the next 22 hours. The "^{3}H-propionic acid" in the urine was measured by extracting 2.0 ml of urine plus 0.2 ml of 5 N hydrochloric acid with 5 ml of ether. Under these conditions 85% of the propionic acid goes into the ether layer. One milliliter of the ether solution was counted in a liquid scintillation counter.

Formic acid was measured chemically by the method of Grant (15) in which formic acid is reduced to formaldehyde by reduction with magnesium metal and the resulting formaldehyde estimated with chromotropic acid. Formic acid was first recovered by distillation from the frozen state of 3 ml of urine which had been acidified to pH 1 to 2 with hydrochloric acid. One-half milliliter of the distillate was placed in a test tube in an ice bath and a coil of 10 cm of magnesium strip added. Preliminary washing of the magnesium with dilute hydrochloric acid was necessary to remove corrosion products which increased the blank. One drop of concentrated hydrochloric acid was added every 2 minutes until 10 drops had been added, the tube being shaken after each drop. Two minutes after the addition of the last drop, the solution was transferred to a 15-ml centrifuge tube with a capillary pipette, leaving the pieces of undissolved magnesium behind. One-and-a-half milliliters of chromotropic acid solution were added. This was prepared fresh every week by dissolving 0.6 g chromotropic acid in 20 ml water and adding 180 ml concentrated sulfuric acid. The tube was covered with a glass marble and placed in a boiling water bath for 30 minutes protected from light. The reaction mixture was stored overnight at 4° to facilitate removal of the precipitate which formed slowly. The solution was centrifuged, and

[3] The authors are indebted to Dr. Mylon Bullock for the preparation of the tritiated propionate.

the supernatant removed and read in a spectrophotometer at 570 mμ. In this procedure 0.2 μmole of formic acid gives an optical density of about 0.3 in a 1-cm cell. Each sample was corrected for the efficiency of freeze-drying distillation from recovery of added ^{14}C-formate.

Formiminoglutamic acid was measured by the enzymatic method of Tabor and Wyndgarden (16).

RESULTS

The addition of 20 or 40 g of calcium propionate markedly depressed growth with the 30% soy protein diets (table 2). However, it does not appear that the growth depression is due primarily to an increased vitamin B_{12} requirement, since the growth increment due to vitamin B_{12} supplementation was the same in the absence or presence of calcium propionate (table 2). This is in contrast with the observation that the addition of propionic acid (9) or sodium propionate (10) to a casein diet would increase the response to vitamin B_{12}. The growth depression produced by calcium propionate was much less with the 70% than with the 30% protein diet.

The excretion of FIGLU with the different types of diets is indicated in table 3. An increase in FIGLU excretion occurred in the absence of vitamin B_{12} with the soy protein diets and in the absence of folic acid with the casein diet. The increased FIGLU excretion developed rapidly with the 30% soy protein diet, rising to 125 μmole in 4 days from a baseline value of 2 to 10 μmole for weanling rats fed a commercial stock diet.

The excretion rate on the 30% soy protein diet without vitamin B_{12} continued to increase with time, reaching a maximum after 29 days. In the 30% soy protein diet supplemented with vitamin B_{12}, there was an appreciable excretion of FIGLU (about 50 μmole/day) during the first 2 weeks which was about 10 times higher than that with the casein diet supplemented with both vitamin B_{12} and folic acid. The FIGLU excretion with the 30% soy protein diet with vitamin B_{12} decreased with time to a level of 1 μmole by the 56th day. The higher FIGLU excretions with the 30% soy protein diet even in the presence of both folic acid and vitamin B_{12} may be due to the lower methionine content of this diet (0.27%) than in the casein diet (0.61%), and also to its slightly higher content of histidine (0.7% in the 30% soy protein diet and 0.45% in the casein diet) which serves as the precursor of FIGLU. This initial rise in FIGLU concentration with the 30% soy protein with vitamin B_{12} and folic acid, followed by a decrease to low levels as the animals grew older, has been observed in other similar experiments. This may represent a decreased requirement for methionine in the larger animal.

By 29 days the FIGLU excretion with the unsupplemented 30% soy protein diet had reached a maximum indicating that the maximal degree of vitamin B_{12} deficiency had been reached as measured by this biochemical criteria. At this time

TABLE 2

Effect of calcium propionate on growth and the supplementary effect of vitamin B_{12}

Basal diet [1]	Ca propionate/kg diet	Avg body wt and survivors at 56 days		Gain due to vitamin B_{12}
		Without vitamin B_{12}	With vitamin B_{12}	
	g	*g*	*g*	*g*
30% Soy protein	—	162(7) [3]	212(8)	50
30% Soy protein	20	112(8)	176(8)	64
30% Soy protein	40	105(8)	87(8)	−18
70% Soy protein	—	224(8)	326(8)	102
70% Soy protein	20	216(8)	302(8)	86
70% Soy protein	40	194(8)	302(8)	108

[1] Basal diet supplemented with 2 mg folic acid/kg diet.
[2] 30 μg vitamin B_{12}/kg diet.
[3] Numbers in parentheses indicate number of survivors.

TABLE 3

Effect of vitamin B_{12} and calcium propionate on excretion of formiminoglutamic acid (FIGLU)

Group no.	Basal diet	Supplement/kg diet			FIGLU excretion/kg body wt/day				Avg body wt	
		Vitamin B_{12}	Folate	Ca propionate	4 Days	15 Days	29 Days	56 Days	29 Days	56 Days
		μg	*mg*	*g*	*μmole*	*μmole*	*μmole*	*μmole*	*g*	*g*
1	30% Soy protein	—	2	—	125	172	400	221	103 [1]	162 [1]
2	30% Soy protein	—	2	20		79			86	112
3	30% Soy protein	—	2	40		30			71	105
4	30% Soy protein	30	2	—	68	51	14	1	123	212
5	30% Soy protein	30	2	20		35			106	176
6	30% Soy protein	30	2	40		7			61	87
7	70% Soy protein	—	2	—	1	45		9	142	224
8	70% Soy protein	—	2	20		48			121	216
9	70% Soy protein	—	2	40		58			105	194
10	70% Soy protein	30	2	—	1	4		2	217	326
11	70% Soy protein	30	2	20		4			196	302
12	70% Soy protein	30	2	40		8			194	302
13	20% Casein	30	–	—		181	450	480	136	184 [1]
14	20% Casein	30	2	—		5	1	2	175	262

[1] Seven survivors.

TABLE 4

Effect of vitamin B_{12}, folic acid and protein levels on metabolism of formate, formiminoglutamic (FIGLU) acid and propionate

Group no.	Basal diet	Supplement/kg diet		Avg body wt at 56 days	^{3}H-propionate excretion	^{14}C-Formate excretion	Endogenous FIGLU excretion/ kg body wt/day	Endogenous formate excretion/ kg body wt/day
		Vitamin B_{12}	Folate					
		μg	*mg*	*g*	%	%	*μmole*	*μmole*
1	30% Soy protein	—	2	162 [1]	3.10	7.6	221	730
4	30% Soy protein	30	2	212	0.28	3.5	1	32
9	70% Soy protein	—	2	224	11.40	8.3	9	125
12	70% Soy protein	30	2	326	0.44	7.6	2	26
15	20% Casein	30	–	184	0.27	18.7	480	1810
16	20% Casein	30	2	262	0.28	4.3	2	23

[1] Seven survivors.

there was a 20% difference in growth between the vitamin B_{12}-deficient animals and those given the supplement, whereas at 56 days there was a 30% difference. The addition of calcium propionate decreased FIGLU excretion rather than increasing it, so there is no evidence as measured by FIGLU excretion that calcium propionate accentuates vitamin B_{12} deficiency.

With the 70% soy protein diet the growth rate was approximately 50% higher than with the 30% soy protein diet. Vitamin B_{12} deficiency was, however, more marked as the growth response due to vitamin B_{12} was 52% with the 70% protein diet at 29 days, as compared with a 20% response with the 30% soy protein diet. Despite the more acute vitamin B_{12} deficiency with the 70% soy protein diet, the excretion of FIGLU was much lower than with the 30% diet. This lower excretion may be a reflection of the higher methionine content (0.7%) in the 70% soy protein diet, since the addition of methionine greatly reduces FIGLU excretion in rats in both vitamin B_{12} and folic acid deficiencies (3). The 70% soy protein diet contains the same amount of methionine (0.70%) as the folic acid-deficient casein diet.

The excretion of endogenous formate and the excretion of a test dose of ^{14}C-formate was measured and the results are shown in table 4. The excretion of endogenous formate approximately paralleled that of FIGLU, both being increased in folic acid deficiency with the casein diet and in vitamin B_{12} deficiency with the 30 and 70% soy protein diets. With the casein diet the excretion of ^{14}C-formate was increased fourfold by folic acid deficiency, whereas with the 70% soy protein diet it was not affected by vitamin B_{12}, and with the 30% soy protein diet it was increased 2 times by vitamin B_{12} deficiency.

The effect of deficiencies of vitamin B_{12} and folic acid on the excretion of a test dose of propionate is shown in table 5. The excretion was measured over a 22-hour period following injection of the test dose. Excretion during the first 22-hour period was essentially complete since the excretion during the second and third days was about 2 and 1%, respectively, of that

TABLE 5

Effect of vitamin B_{12}, folic acid, and protein level on excretion of an intraperitoneal dose of tritiated propionic acid

Group no.	Basal diet	Supplement/kg basal diet		Excretion of ^{3}H-propionate			Avg body wt		
		Vitamin B_{12}	Folate	14 Days	28 Days	56 Days	14 Days	28 Days	56 Days
		μg	*mg*	%	%	%	*g*	*g*	*g*
1	30% Soy protein	—	2	0.35	1.03	3.10	71	103 [1]	162 [1]
2	30% Soy protein	30	2	0.21	0.27	0.28	73	123	212
5	70% Soy protein	—	2	5.70	11.20	11.40	88	142	224
6	70% Soy protein	30	2	0.37	0.37	0.44	105	217	326
15	20% Casein	30	—			0.27	83	136	184
16	20% Casein	30	2			0.28	97	175	262

[1] Seven survivors.

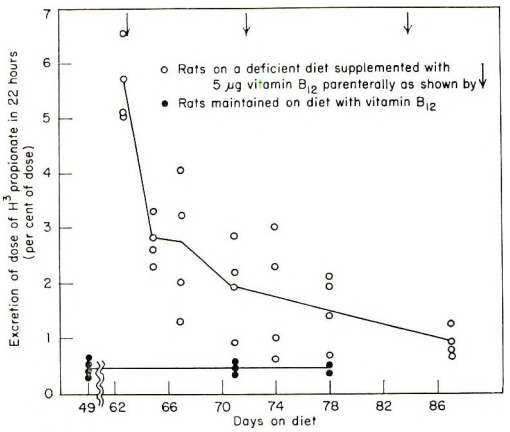

Fig. 1 Effect of vitamin B_{12} in reducing excretion of a parenteral dose of ^{3}H-propionate.

amount excreted on the first day. The results showed that the excretion of a test dose of propionate was increased in vitamin B_{12} deficiency but not in folic acid deficiency. With the 30% soy protein diet there was no difference at 14 days between diets containing, and those not containing, vitamin B_{12}. At 28 days the excretion of propionate in the absence of vitamin B_{12} had increased threefold and at 56 days had increased tenfold. With the 70% soy protein diet the excretion of propionate in the absence of vitamin B_{12} had increased to 15 times that of the supplemented group and by 28 days to 30 times.

When vitamin B_{12} was administered, the excretion of administered propionate decreased slowly, requiring 4 weeks to return to normal, as shown in figure 1. Four rats that had been fed the 30% soy protein diet, vitamin B_{12}-deficient, for 62 days were given 5 μg vitamin B_{12} intraperitoneally. Their excretion of administered propionate was 5.7% prior to the administration of vitamin B_{12}, and this decreased to 2.75% 2 days after giving vitamin B_{12}, and to 1.8% 8 days later. The excretion still remained 3 times higher than normal after 15 days, and 24 days were required for complete normalization of propionate excretion.

DISCUSSION

Although vitamin B_{12} is not directly involved in the metabolism of either FIGLU or formate, it can influence the excretion rates of both. The excretion of formate roughly parallels that of FIGLU and is five to ten times as high. It appears that the excretion of formate offers little advantage over FIGLU as a diagnostic aid for the differentiation between vitamin B_{12} and folic acid deficiency. Although formate and FIGLU excretions parallel each other in the conditions studied here, their metabolic origins are different. The formate excreted in folic acid deficiency is not derived from breakdown of FIGLU but may be derived in part from tryptophan, since the feeding of tryptophan markedly increases the excretion of formate with a folic acid-deficient diet (2).

The similarity of effect of vitamin B_{12} and folic acid on the excretion of both formate and FIGLU suggests that the effect of vitamin B_{12} may be an indirect one exerted by modification of folic acid metabolism. This finds support in the effect of vitamin B_{12} in decreasing the proportion of 5-methyl tetrahydrofolic acid (5-methyl FH_4) in tissues. Herbert and Zalusky (17) have observed an increase in the proportion of 5-methyl FH_4 relative to other forms of folate in blood in pernicious anemia. The administration of vitamin B_{12} decreases the relative proportion of 5-methyl FH_4 and increases the proportion of other forms of folate. An increase in the relative proportion of 5-methyl FH_4 has also been observed in livers of vitamin B_{12}-deficient rats, and this can be decreased by giving either vitamin B_{12} or methionine (18). It has been suggested by Herbert and Zalusky (17) that 5-methyl FH_4 is not available for certain folic acid coenzyme functions such as metabolism of FIGLU until the methyl group has been removed by the transfer of the methyl group to homocysteine to give methionine by means of a vitamin B_{12}-dependent reaction. The methyl group of methionine is readily oxidized (19) (8 to 50% in 6 hours) in a reaction not dependent on folic acid. Thus the transfer of the methyl group of 5-methyl FH_4 to homocysteine and subsequent oxidation to carbon dioxide may represent a mechanism for regenerating FH_4 and making it available for metabolism of FIGLU. Such a mechanism is supported by the observation that feeding methionine decreases the propor-

tion of 5-methyl FH_4 in the liver (18), and also markedly decreases FIGLU excretion in both folic acid and vitamin B_{12} deficiencies (2–4).

The failure of vitamin B_{12} to prevent the growth depression produced by 2 and 4% of calcium propionate is at variance with the observations of Hogue and Elliot (10) that 3 and 6% sodium propionate produced a growth depression with a casein-sucrose diet which could be partially prevented by vitamin B_{12}. Gammon and Agranoff (20) have observed an increased mortality produced by feeding 10% tripropionin with a casein-sucrose vitamin B_{12}-deficient diet. Since a soy protein diet was used in the experiments reported here, the possibility exists that the difference in response may be in part due to the difference in the basal diet used.

The increase in propionate excretion following injection of a test dose in vitamin B_{12} deficiency is in accord with the known role of vitamin B_{12} in isomerization of methylmalonyl CoA to succinyl CoA in *Propionibacterium shermanii* (21), *Ochromonas malhamensis* (22), rat liver (11) and sheep liver (12). A decreased clearance rate of propionic acid from blood of vitamin B_{12}-deficient sheep (12) has been observed. Methyl malonic acid has been observed in increased concentration in the urine of vitamin B_{12}-deficient rats (23) and in patients with pernicious anemia (24–26).

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Intestinal Absorption of Homologous Lactic Dehydrogenase Isoenzymes by the Neonatal Pig [1,2]

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ABSTRACT Enzymatic activity is generally more susceptible to alteration than other measurable characteristics of a protein. The present experiments were designed to test whether it was possible to feed enzymes to neonatal pigs and have the enzymatic activity survive the alimentary secretions and the passage through the intestinal epithelium. Absorption of enzymes may have some relevance with respect to postnatal maturation. The well-characterized isoenzymes of lactic dehydrogenase (LDH) were the ones followed in this study. Homogenates of adult porcine liver, heart muscle, and skeletal muscle (each with a distinct isoenzyme electrophoretic pattern) were fed to neonatal pigs. The criterion for absorption was an early and marked increase in the blood serum LDH activity of piglets fed tissue homogenates plus a shift of the isoenzyme serum electrophoretic pattern toward that of the fed tissue. Such changes in total activity and electrophoretic pattern were observed in our piglets fed the homogenates versus controls fed a salt solution. It was concluded that the young of certain species can absorb proteins with retained enzymatic activity.

Previously it was shown that piglets are born immature or fetal-like with respect to their serum protein profile (1). The sow via her colostral and milk secretions affects profoundly the development of a mature serum protein profile. Early changes in the piglet's serum proteins result from the absorption through the piglet's gut of proteins that appear to be unaltered with respect to the electrophoretic and serological character. This primitive mechanism for protein absorption is qualitatively non-selective and occurs via an immature intestinal epithelium (2). The maturation of this epithelium also is dependent upon diet (3).

Enzymes have been observed in the colostrum of many species of animals (4). This, coupled with the above-mentioned facts that the lacteal secretions in a special way influence postnatal maturation, raises the question of whether colostral enzymes play a role in rapid neonatal development. It is known that some of the enzymes involved in the metabolism of newborn pigs are present in the sow's colostrum (5).[4] However, before assessing the postnatal influence of colostral enzymes, first it must be learned whether enzymatic activity can survive alimentary secretions and passage through the cells of the neonatal intestinal epithelium. The answer to this question is the subject of this report.

As a model system to test the absorption of biologically active enzyme via intestines, lactic dehydrogenase and the neonatal pig were chosen. Lactic dehydrogenase (LDH) was chosen because it is well characterized and exists in 5 electrophoretic forms (isoenzymes) in the various tissues of the pig as well as in other mammalian species (6). The 5 isoenzymes vary in relative proportions characteristic of the tissue source. Thus, it should be relatively easy to detect absorption in the piglet fed a particular LDH isoenzyme electrophoretic pattern by noting an early and marked increase in the piglet's blood serum LDH activity plus a shift of the serum LDH isoenzyme electrophoretic pattern toward the pattern of the fed tissue. The neonatal pig was

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[2] Presented in part at the 49th Annual Meeting of the Federation of the American Societies for Experimental Biology, Atlantic City, New Jersey, 1965.

[3] Rockefeller Foundation Fellow.

[4] Unpublished data, I. R. Balconi and J. G. Lecce, 1964.

chosen because of his known proclivity to absorb proteins (2).

EXPERIMENTAL

A total of 24 piglets was used. All animals were farrowed in an isolation unit and caught at birth in clean towels, transferred to another isolation unit, caged individually, and assigned at random to one of four experimental diets. Within 4 hours of birth (zero-hour of experimental period) and at 5, 10, and 20 hours thereafter, 3 to 5 ml of blood were withdrawn from the anterior vena cava of each animal. The serum was separated by centrifugation and frozen at −40° until analysis was performed for enzymatic activity. The diets were fed from a pan according to the following schedule: 25 ml immediately after the first bleeding and 40 ml at hourly intervals for the next 10 hours. A total of approximately 425 ml was offered to each animal. After the 10-hour blood sample was taken, the animals were not allowed food or water.

Preparation of diets. Diets were prepared by homogenizing adult porcine tissues in the ratio of one part tissue to 4 parts (w/v) 0.05 M tris hydroxymethylaminomethane·HCl (tris·HCl buffer) at 2° and at pH 7.4. Diets of homogenates of porcine liver, heart, and skeletal muscle were made and stored at −40° until they were used. To minimize loss of enzymatic activity, diets were thawed immediately before feeding the piglets. Total LDH activity and isoenzyme electrophoretic pattern were determined for the supernatant from each diet. The control group received a salt solution containing 2.6 g of KCl; 0.6 g of KH_2PO_4; 3.9 g of $Na_2HPO_4 \cdot 7 H_2O$; and 0.725 g of $MgSO_4 \cdot 7 H_2O$/1000 ml of distilled water.

Determination of LDH isoenzyme electrophoretic pattern. Electrophoresis to separate the LDH isoenzymes was carried out on 75 × 25 mm microscope slides upon which 2 ml of 0.85% agar[5] in pH 8.6 barbital buffer, 0.075 ionic strength had been placed. The electrophoretic technique and apparatus have been described previously (7). Samples from 0.005 to 0.01 ml of the tissue supernatant solution and from 0.03 to 0.04 ml of blood serum were subjected to electrophoresis at 26° to 30° for 48 minutes with a potential drop of 90 volts. Temperature was maintained by immersing the slides during electrophoresis in cooled varsol. After electrophoresis, the slides were incubated for 60 minutes in the dark at 37° in the following substrate solution (modified from Wieme et al.) (8): 25 mg of nitroblue tetrazolium;[6] 15 mg of phenazine methosulfate;[7] 60 mg of NAD;[8] 1 ml of KCN solution (1 mg/ml of water); 1 ml of 1 M sodium lactate; 1.9 g of $Na_2HPO_4 \cdot 2 H_2O$; 25 mg of KH_2PO_4; and distilled water to a total volume of 90 ml. This solution was prepared 30 minutes before immersing the slides, with the exception of phenazine methosulfate which was added immediately before placing the slides in the solution.

After the incubation period, slides were washed in 2% acetic acid for 4 hours and dried on a warming plate at 50°. The percentage of relative activity of the LDH isoenzymes separated by electrophoresis and visualized in the way described above was determined with a recording microdensitometer scanning at 525 mμ and equipped with an electronic integrator.[9]

The units per milliliter of LDH activity were determined from the change in absorbancy during the conversion of $NADH_2$ into NAD at 340 mμ at 25° for 1 minute at 15-second intervals or for 5 minutes at 1-minute intervals (9). The substrate solution consisted of 2.9 ml of 0.1 M phosphate buffer pH 7.1; 0.03 ml of 0.02 M sodium pyruvate; 0.02 ml of 0.1 M potassium cyanide solution in distilled water; 0.01 ml of tissue supernatant or 0.05 ml of blood serum. This mixture was allowed to equilibrate at room temperature (25°) for 10 minutes and then the reaction was initiated by the addition of 0.03 ml of $NADH_2$ (10 mg/ml of glass-distilled water). A unit of LDH is defined as that amount of enzyme per milliliter required to effect a decrease of 0.01 in absorbancy per minute under the assay conditions stated above.

Data were expressed as absolute LDH activity in units per milliliter and as rela-

[5] Consolidated Laboratories, Inc., Chicago Heights, Illinois.
[6] Nutritional Biochemicals Corporation, Cleveland.
[7] See footnote 6.
[8] See footnote 6.
[9] Photovolt Corporation, New York.

tive LDH activity in per cent of each isoenzyme.

RESULTS

Porcine LDH electrophoretic pattern. Figure 1 shows the isoenzyme pattern of adult skeletal muscle, heart and liver. Skeletal muscle is rich in the slow-moving LDH_5; heart is rich in the fast-moving LDH_1, whereas liver with LDH_1, LDH_2 and LDH_3 contains hybrids [10] of both. Homogenates are easily distinguished from each other by their LDH isoenzyme pattern.

Control group. In this trial, the LDH isoenzymes in blood serum of neonatal pigs fed a salt solution from zero to 10 hours showed a slight decrease in the absolute activity of LDH_1 (decreased from 60.0 to 49.1%) and minor changes in the remaining LDH fractions (fig. 2). The LDH activity in the serum in units per milliliter remained rather constant varying between 38 and 45. The stability of the serum LDH isoenzyme pattern during this 20-hour period expressed as absolute activity (relative per cent of each band × units per ml) is shown in figure 2.

Group fed liver homogenate. In the second trial, 6 piglets were fed a homogenate of liver with 700 LDH units/ml of the supernatant and an LDH isoenzyme pattern of mainly LDH_1, LDH_2 and LDH_3 (fig. 3A). Ten hours after the first feeding, all animals in this group showed an increase in LDH activity from an average of 41 to 147 LDH units/ml of serum. At 20 hours (10 hours after the last feeding of the homogenate), the average LDH units per milliliter of serum was 123 as compared with an average of 38 LDH

[10] Appella, E., and C. L. Markert 1962 Physicochemical properties of purified isozymes of lactate dehydrogenase from different sources. Federation Proc., *21*: 253 (abstract).

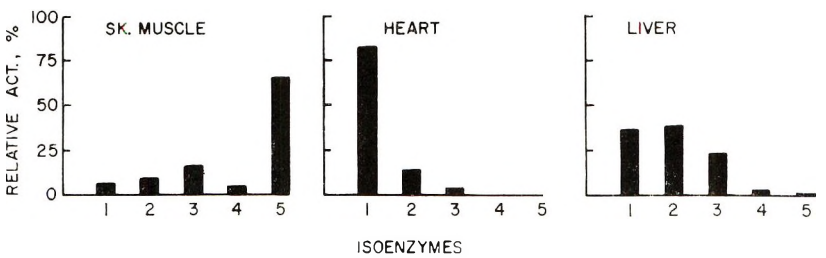

Fig. 1 Lactic dehydrogenase electrophoretic isoenzyme pattern of homogenates of adult pigs' skeletal muscle, heart and liver.

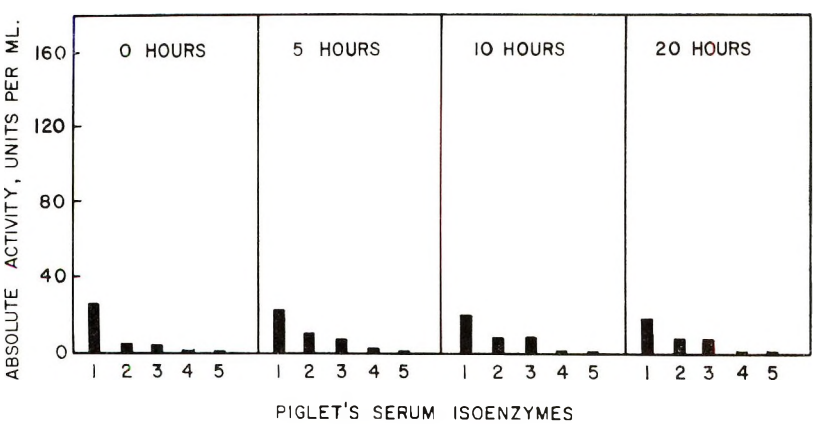

Fig. 2 Average changes in serum lactic dehydrogenase electrophoretic isoenzyme pattern in 6 control piglets fed 425 ml of a salt solution from zero to 10 hours. Piglets were bled at zero, 5, 10 and 20 hours.

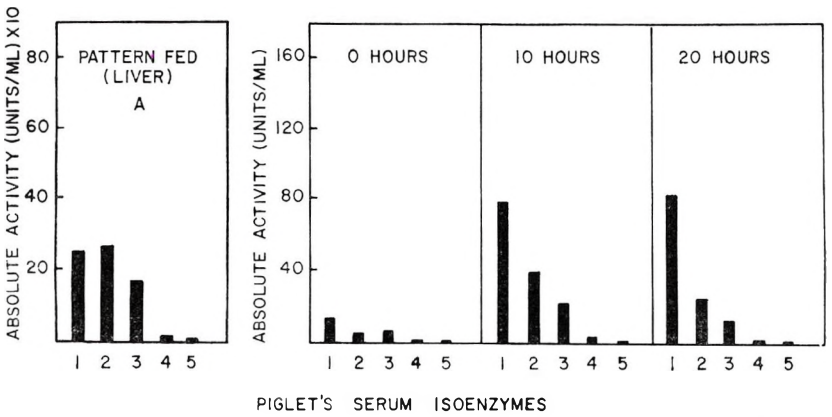

Fig. 3 Average changes in serum lactic dehydrogenase electrophoretic isoenzyme pattern in 6 piglets fed 425 ml of homogenate of liver containing 700 LDH units/ml of supernatant. Piglets were fed from zero to 10 hours and bled at zero, 5, 10 and 20 hours.

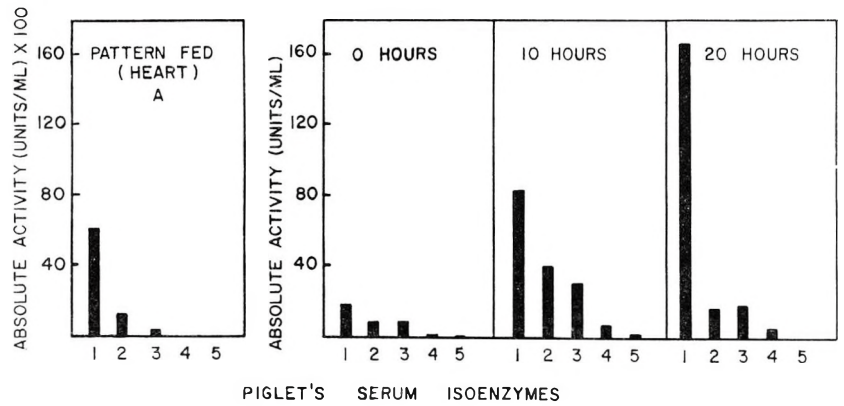

Fig. 4 Average changes in serum lactic dehydrogenase electrophoretic isoenzyme pattern in 6 piglets fed 425 ml of a homogenate of heart containing 7,375 LDH units/ml supernatant. Piglets were fed from zero to 10 hours and bled at zero, 5, 10 and 20 hours.

units/ml of serum in the controls of the same age.

Accompanying the increase in serum LDH activity was an increase in only those isoenzymes predominating in the diet (LDH_1, LDH_2 and LDH_3). This information is plotted as changes in absolute activity (relative per cent of each fraction $\times$ units per ml in serum) in figure 3.

Group fed heart muscle homogenate. Six piglets were fed a homogenate of heart containing 7,375 LDH units/ml and an LDH isoenzyme pattern in which LDH_1 was the major fraction followed by small amounts of LDH_2 and LDH_3 (fig. 4A). Five hours after the first feeding of homogenate, again the piglets' serum showed only slight changes in LDH activity. However, at 10 hours the average total LDH activity for this group increased to 164 units/ml of serum as compared with 42 units/ml of serum in the controls of the same age. Accompanying the increase in total LDH activity was an increase in the relative activity of the 3 isoenzymes present in the homogenate, LDH_1, LDH_2 and LDH_3. At 20 hours the average total LDH activity was 207 units/ml of serum (38 units per ml in the serum of the controls) with a marked increase in LDH_1 (fig. 4).

Group fed skeletal muscle homogenate. In the fourth trial, a homogenate of skele-

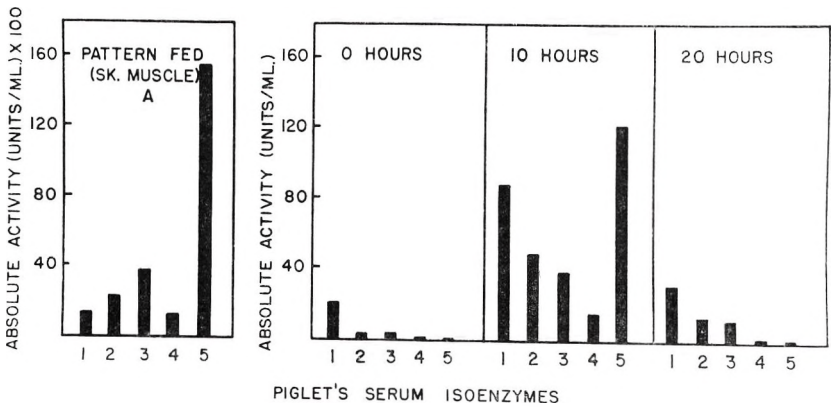

Fig. 5 Average changes in serum lactic dehydrogenase electrophoretic isoenzyme pattern in 6 piglets fed 425 ml of homogenate of skeletal muscle containing 23,400 LDH units/ml of supernatant. Piglets were fed from zero to 10 hours and bled at zero, 5, 10 and 20 hours.

tal muscle containing 23,400 LDH units/ml of supernatant and rich in LDH_5 was fed to 6 piglets (fig. 5A). Five hours after first feeding the homogenate, the average total LDH activity for this group increased to 134 units/ml of serum. Simultaneously, LDH_5 appeared in the serum of the piglets. At 10 hours the total LDH activity for this group increased to 312 units/ml of serum accompanied by a marked increase in the relative activity of LDH_5.

At 20 hours, the activity in the sera of all pigs returned to near-normal levels for this age. The average total LDH activity for the group was 62 units/ml and the LDH isoenzyme pattern resembled the controls of the same age (fig. 5).

DISCUSSION

Neonates of some animal species (pigs included) can absorb macromolecules for a short period of time after birth (2, 10). These macromolecules are presented to the neonate via the dam's first mammary secretions, called colostrum. Macromolecules of varied biological activity are observed in colostrum. One such is γ-globulin with antibody activity. Another is hormone with regulatory activity; and still another is enzyme with catalytic activity. It is known that γ-globulin survives the gastrointestinal environment of some neonates and is absorbed fully active (10). Insulin was shown to do likewise (11–13). Aside from a report by McCance et al. (14), who noted an increase in cholinesterase in puppies eating bitch's colostrum rich in cholinesterase, and a contrary report by Halliday and Mihailovic (15) using suckling rats, little information is available concerning the resistance of enzymatic activity to the intestinal milieu. Results recorded herein show that serum from neonatal piglets fed a diet rich in lactic dehydrogenase had (a) a marked increase in lactic dehydrogenase, and (b) a shift in the serum lactic dehydrogenase isoenzyme pattern toward the pattern found in the diet. This was true whether the diet source was heart muscle (high in LDH_1), skeletal muscle (high in LDH_5), or liver (high in LDH_1, LDH_2 and LDH_3). These data are interpreted as evidence that enzymatic activity can survive neonatal alimentary secretions and passage through the intestinal epithelium.

The changes in the neonate's serum isoenzyme pattern toward that of the fed tissue, whether it was high in LDH_1 or LDH_5 or combinations thereof, suggests that all 5 isoenzymes are absorbed by the neonatal pig. Whether all LDH isoenzymes are absorbed to the same degree must be determined quantitatively in future experiments. A comparison of the 20-hour serum isoenzyme pattern of piglets fed a homogenate rich in LDH_1 to that of piglets fed a homogenate rich in LDH_5 shows a rapid decline in LDH_5, while LDH_1 remains elevated. This could result from an inactivation of LDH_5 in the blood or from a selective absorption of LDH_5 by organs

such as spleen and liver. Future experiments will dwell on this point.

Whether macromolecules in the form of absorbed enzymes have a role in the health, vigor and development of the neonate remains conjecture at the moment. But it is not difficult to imagine situations where absorbed enzymes would have survival value — for example, absorption of large amounts of lactic dehydrogenase would be an advantage in the event of lactic acidosis (16), or absorption of cholinesterase in an exposure to insecticide.

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Plasma Amino Acid Levels of Men Fed Diets Differing in Protein Content. Some Observations with Valine-deficient Diets [1]

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ABSTRACT The time course of alterations in plasma amino acid patterns developing during the ingestion of a low protein diet was investigated and attempts were made to correlate the changes with tissue nitrogen loss. Post-absorption plasma amino acid levels were measured at intervals during a 5-week period in 6 men aged 55 years or older who were receiving a diet containing 3.5 g of nitrogen/day. The essential amino acid values decreased throughout the period with valine showing a significant reduction after 1 week as compared with values obtained when the subjects were given a diet of 14 g of nitrogen/day. The total nonessential amino acid levels in plasma increased after 1 week of administering the low protein diet and showed no further change. The increase was due to higher values for glutamine-asparagine, glycine and alanine. During the 5-week period, the ratio of essential to nonessential amino acids decreased from a mean of 0.43 to 0.28 and this decrease was associated with a body nitrogen loss of from 30 to 75 g. Reducing the daily caloric content of the low protein diet from 2500 kcal to 900 kcal did not appear to alter the plasma amino acid response. When a valine-deficient diet was given to subjects, the plasma valine levels during post-absorption were greatly decreased within 5 days.

The advent of ion exchange chromatography methods for the separation and determination of amino acids has made possible a more systematic investigation of the effects of the dietary protein content on plasma amino acid levels. The goal can now be established of ascertaining whether plasma amino acid patterns can be used in the evaluation of protein nutritional status. Preliminary investigations (1, 2) with adult men who ingested diets low in protein but adequate in calories and other nutrients have shown that the total amount of the essential amino acids in plasma is decreased, whereas the total for the nonessential amino acids is increased. Alterations of plasma amino acid patterns in children with kwashiorkor have also been reported (3–5). Synderman and associates (3) have shown that the ratio of tyrosine to phenylalanine is decreased and suggest the use of this ratio in the diagnosis of protein deficiency. Whitehead (5) observed reductions in some of the essential amino acids and increases in the nonessential amino acids of plasma in cases of subclinical kwashiorkor.

The present report describes the results of measuring the plasma amino acid levels during post-absorption in men receiving low protein diets. The plasma amino acids were determined at intervals during a period of 5 weeks or longer when the subjects received a diet containing 3.5 g of nitrogen/day and maintained a negative N balance throughout the period. These results are compared with determinations made on the same individuals when they were ingesting diets containing 14 g of N/day. Studies were also conducted with diets supplying 7 g of N daily and with low calorie diets. One subject was maintained for 8 days with a protein-free diet adequate in calories. In addition, the results obtained with feeding a semi-purified diet containing purified amino acid mixtures devoid of valine are given.

EXPERIMENTAL PROCEDURE

Subjects. Twelve men ranging in age from 57 to 69 years and in weight from

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[1] Supported by Public Health Service Research Grant no. AM-01347 from the National Institute of Arthritis and Metabolic Diseases.

[2] Deceased.

63.5 to 77 kg participated in various parts of this study. They were judged to be in good health on the basis of a thorough medical examination. During their association with the study, they resided in a metabolic ward under close clinical and nursing supervision. The subjects ate all their meals in the ward, but were ambulatory during the day and engaged in hobbies and other recreation.

Laboratory methods. The dietary and N balance techniques were the same as in previous studies (6, 7). Blood for amino acid analyses was withdrawn from the antecubital vein of subjects who had been without food for 12 to 14 hours. The blood was processed according to the procedures of Tallan et al. (8) and the amino acids were measured in picric acid extracts of plasma by ion exchange chromatography using a Spinco-Beckman analyzer. Statistical summaries of the results obtained were carried out with a computer.[3]

Diets. Except for 2 subjects who received diets containing purified amino acid mixtures varying in valine content, all subjects were fed diets of ordinary food containing either 3.5, 7, or 14 g of N/day. The composition of these diets and their effects on N balance have been dealt with in previous communications (6, 7). The diets with 7 g of N and 14 g of N contained protein from meat and dairy products, and the subjects were able to maintain N balance when ingesting these diets. The diet with 3.5 g of N contained protein as vegetables and cereals, except for 50 g of whole milk. It has been found that subjects of this age group do not maintain N balance with this diet, although they achieve N equilibrium with an intake of 3.5 g of N when the chief source of N is egg protein (1).

The amino acid content of the diets containing 3.5 and 14 g of N were determined in acid hydrolysates (an aliquot of homogenized diet was hydrolyzed in an evacuated tube in 6 N HCl for 22 hours at 110°) by ion exchange chromatography. The diet with 3.5 g of N had the following essential amino acid content: (g/day) threonine, 0.7; valine, 1.0; methionine, 0.23; isoleucine, 0.8; leucine, 1.5; tyrosine, 0.22; phenylalanine, 0.9; and lysine, 0.7, for a total of 6.1 g. Tryptophan and cystine were not determined but the amount of cystine as calculated from tables (8) was 0.22 g. In the diet with 14 g of N, the essential amino acid content was as follows (g/day) threonine, 3.9; valine, 3.6; methionine, 1.0; isoleucine, 2.6; leucine, 5.6; tyrosine, 0.6; phenylalanine, 2.4; and lysine, 5.4, for a total of 25.1 g. The amount of cystine was calculated to be 1.2 g. The total amounts of the nonessential amino acids in the low protein and high protein diets were 12.8 and 41.3 g/day, respectively. Aspartic acid plus glutamic acid constituted approximately 50% of the total amounts of nonessential amino acids for both diets.

For preparing the valine-deficient diet, the purified amino acid mixtures (7) that were used were patterned after the amino acid proportions of egg protein. The essential amino acids were added in amounts equivalent to the quantities present in 150 g of egg. Other dietary sources consisting of low protein fruits and vegetables contributed approximately 0.4 g of N and the nonessential amino acids were added in amounts to bring the total N of the diet to 7 g/day.

Except for a few instances where 900 kcal/day were fed, the calories were adjusted for each individual to maintain the body weight. With the subjects on this study, the caloric intake varied from 2100 to 2500 kcal/day. When 2 subjects were fed the amino acid mixtures, their caloric intakes were increased by approximately 10%.

RESULTS

In table 1 are recorded the mean values for the acidic and neutral amino acids in blood plasma obtained during post-absorption from 6 subjects who received food varying in N content during alternate dietary periods. The general procedure was to administer the low protein diet of 3.5 g of N first and then to substitute the diet containing 14 g of N for a subsequent period. All subjects had been receiving regular hospital diets calculated to contain from 13 to 18 g of N/day with an average value of 15 g for at least 2 weeks prior to the study. Because of the difficulties in-

[3] Acknowledgment is given to the UCLA Health Science Computer Facility for use of the computer.

TABLE 1

Plasma amino acid values of subjects receiving diets containing 3.5, 7.0, or 14.0 g of N per day [1]

Amino acid	N content of diet and period of administration					
	3.5 g N/day				14 g N/day [2]	7 g N/day [3]
	Zero week (a)	1 week (b)	3 weeks (c)	5 weeks (d)	1 week (e)	1 week (f)
	μmole/100 ml	*μmole/100 ml*	*μmole/100 ml*	*μmole/100 ml*	*μmole/100 ml*	*μmole/100 ml*
Essential [4]						
Threonine	12.0 ± 1.4	10.9 ± 1.6	9.4 ± 1.4	8.3 ± 1.1	9.9 ±1.0	10.7 ± 2.7
Valine	18.2 ± 0.9	14.3 ± 2.1*	13.7 ± 2.2**	12.3 ± 1.0*	17.8 ±2.7	16.5 ± 2.2
½ Cystine	10.8 ± 2.1	10.7 ± 1.9	10.1 ± 1.3	9.6 ± 1.6	9.9 ±1.3	10.2 ± 1.8
Methionine	2.6 ± 0.2*	2.5 ± 0.3*	2.3 ± 0.3*	2.0 ± 0.1*	1.8 ±0.1	2.1 ± 0.5
Isoleucine	5.9 ± 0.6	5.3 ± 1.3	5.3 ± 1.2	4.8 ± 0.6	5.8 ±0.5	5.5 ± 1.2
Leucine	10.5 ± 0.9	9.0 ± 1.1	8.9 ± 1.2	7.9 ± 0.4	9.5 ±1.3	9.7 ± 1.8
Tyrosine	5.6 ± 1.0	5.1 ± 1.0	5.0 ± 1.2	4.5 ± 1.0	4.7 ±0.9	5.1 ± 1.3
Phenylalanine	5.1 ± 0.6	4.3 ± 0.5	4.2 ± 0.5	3.7 ± 0.4	4.1 ±0.6	4.3 ± 1.2
Total	70.7 ± 4.1	62.2 ± 7.2	58.7 ± 7.9	53.1 ± 3.7	63.4 ±5.7	64.1 ±10.7
Nonessential						
Serine	12.2 ± 1.3	11.2 ± 2.2	10.5 ± 2.3	9.2 ± 2.0	10.1 ±0.7	10.5 ± 0.9
Glutamine + asparagine	60.1 ± 5.4	78.6 ± 8.4**	79.0 ±16.9	78.6 ± 9.6**	63.4 ±7.0	64.3 ±13.8
Proline	15.6 ± 1.9	16.0 ± 3.2	16.2 ± 2.1	15.8 ± 2.0	15.6 ±1.3	17.1 ± 3.2
Glutamic acid	8.6 ± 3.7	7.7 ± 2.3	7.6 ± 2.8	8.5 ± 3.0	6.6 ±1.6	8.5 ± 4.5
Citrulline	4.6 ± 1.1	3.5 ± 1.5	4.2 ± 1.0	3.8 ± 0.3	4.3 ±0.5	3.8 ± 0.8
Glycine	24.4 ± 2.8	27.7 ± 2.3*	26.8 ± 1.8**	25.5 ± 2.3*	19.7 ±2.0	21.8 ± 4.3
Alanine	33.4 ±10.4	46.9 ±16.8*	46.7 ±13.3*	50.4 ±12.3*	27.5 ±4.9	35.2 ± 7.6
α-NH_2-N-butyric acid	1.2 ± 0.4	0.7 ± 0.2*	0.7 ± 0.3*	0.7 ± 0.3**	2.2 ±0.7	1.5 ± 0.8
Total	160.1 ±17.5	192.3 ±25.1*	191.7 ±20.6*	192.5 ±21.1**	149.5 ±7.3	162.7 ±23.6
E/N ratio [5]	0.45± 0.05	0.33± 0.04**	0.31± 0.06**	0.28± 0.03**	0.43±0.05	0.40± 0.05

[1] Mean of acidic and neutral amino acids ± SD for the same 6 subjects except for the zero-week value where the mean is for 4 of the 6 subjects and the 7 g N intake where the mean is for 6 subjects, 3 of whom were not fed the other diets.
[2] Administered following the diet containing 3.5 g N/day.
[3] Administered following a hospital diet calculated to contain an average of 15 g N/day.
[4] Includes cystine and tyrosine.
[5] For acidic and neutral amino acids.
*, ** Significant difference in means of paired differences from values for the 14 g N intake at 1.0% and 0.1% levels, respectively, by *t* test.

volved in feeding these subjects controlled diets for prolonged periods of time, only four of the six subjects were given the diet with 14 g of N for 4 to 7 days before administration of the low protein diet. The plasma amino acid values for these subjects are shown in the table in column *a*. Except for methionine, the values were not significantly different at the 1% level from the values obtained when the subjects received 14 g of N/day (column *e*) for one week following the period with the low protein diet. It appears that in this group of subjects, methionine levels did not respond as rapidly as the other essential amino acids in plasma to an increase in dietary protein. Five of the subjects were given the diet with 14 g of N for a period of time longer than one week. However, the values for the succeeding time periods were very similar to those of column *e* and hence are not recorded in the table. After 2 weeks of the 14-g N intake, the average value for the total essential amino acids in plasma was 64.9 μmole/100 ml and for the total nonessential amino acids, 145.7 μmole with an E/N ratio of 0.45.

Plasma amino acid values are also given for 6 subjects who received a diet containing 7 g of N/day (table 1, column *f*). This diet was not administered in sequence with the other 2 diets, but was part of another metabolic study. However, 3 subjects participated subsequently in the investigation with the 3.5- and 14-g levels of N intake. None of the mean plasma amino acid values obtained when subjects were receiving the diet with 7 g of N were significantly different from the values found with the 14-g N intake (column *e*).

As the subjects continued to ingest the diet containing 3.5 g of N, the mean essential amino acid levels in plasma consistently decreased and for the most part, the values for each subject also showed continued reductions throughout the 5 week period (table 1, columns *b*, *c* and *d*). However, only the valine value showed a significant decrease during this time period from values obtained when the subject received a diet of 14 g of N.

A dichotomy developed in the response of the nonessential amino acids in plasma when the low protein diet was administered. Some were either reduced or remained at approximately the same level, but others were increased. The α-NH_2-*n*-butyric acid, a metabolite of threonine and methionine, showed a significant reduction from the value obtained when the subjects ingested the diet containing 14 g of N. Serine, proline, citrulline and glutamic acid remained relatively constant. But alanine, glutamine plus asparagine, and glycine were significantly higher than levels observed when the subjects received the high protein diet. Since in terms of quantity these amino acids represent a major component of the nonessential amino acids, the total value for these amino acids was significantly increased (column *b* vs. column *e*). The increase occurred after one week of low protein intake and showed no further change. This is in contrast with the results with the total essential amino acids as these values continued to decrease throughout the period the low protein diet was being fed.

As a consequence of these changes in plasma amino acid levels, the ratio of the essential to the nonessential acidic and neutral amino acids (E/N ratio) was lower in subjects when they were ingesting the low protein diet (0.33 at 1 week) than when they were receiving the diet with 14 g of N (0.44). The mean E/N ratio continued to decrease throughout the low protein dietary period as a result of the continued reduction in essential amino acid values.

The basic amino acids in plasma were not determined for all subjects for all time periods and hence they are not included in the table. The mean lysine values obtained for 4 subjects receiving the low protein diet for 5 weeks was 16.4 ± 2.8 μmole/100 ml and for the same subjects receiving the diet with 14 g of N for one week, the mean value was 17.1 ± 1.8 μmole. The corresponding values for the nonessential amino acids were as follows (in μmole/100 ml with the value for the low protein diet being listed first): ornithine, 7.5 ± 1.5 vs. 7.1 ± 2.0; histidine, 6.2 ± 0.2 vs. 7.1 ± 0.5; arginine, 8.2 ± 0.4 vs. 6.5 ± 1.4. None of these values was significantly different. The E/N ratios shown in table 1 represent only the acidic and neutral amino acids, but E/N ratios calculated to include the basic amino acids are almost

identical, averaging only 0.05 higher with both the high and low protein diets and therefore these ratios can be used interchangeably, at least under conditions of this study.

As was expected, the plasma urea values reflected the protein content of the diet. The mean values for subjects ingesting the diet with 14 g of N was 300 ± 31 μmole/100 ml and after 5 weeks of the low protein diet, 127 ± 55 μmole.

For two of the six subjects, the diet containing 3.5 g of N was administered for a second time after an interval during which the diet with 14 g of N was fed. In both subjects, re-feeding the low protein diet again resulted in a low plasma E/N ratio with a decrease in the essential amino acids and an increase in the nonessential amino acids (these results are not tabulated).

In table 2 are shown the mean plasma amino acid values for 2 subjects who received the low and high protein diets for extended periods of time. The values represent repeated determinations made at 5-day intervals after 5 weeks of ingesting the low protein diet and after 4 weeks of ingesting the diet containing 14 g of N. These results provide additional evidence that low and high protein intakes are associated with different and characteristic plasma amino acid patterns which are reflected in the E/N ratio. The standard deviations recorded in table 2 are of the same order of magnitude as those of table 1, an indication that intra-subject variation in levels of individual amino acids is comparable to inter-subject variation even after prolonged periods of controlled dietary intake.

TABLE 2

Plasma amino acid values obtained from 2 subjects after they had received the diet with 3.5 g of N for at least 40 days and the diet with 14 g of N for at least 30 days [1]

Amino acid	Subject 1		Subject 4	
	Diet with 3.5 g of N	Diet with 14 g of N	Diet with 3.5 g of N	Diet with 14 g of N
	μmole/100 ml	*μmole/100 ml*	*μmole/100 ml*	*μmole/100 ml*
Essential [2]				
Threonine	7.2 ± 1.0**	11.6 ±0.7	6.7 ±0.4*	11.1 ±0.9
Valine	11.9 ± 1.4**	19.1 ±1.3	12.5 ±1.7	19.0 ±0.7
½ Cystine	8.4 ± 1.1	8.9 ±1.7	9.0 ±1.2	10.5 ±0.9
Methionine	2.2 ± 0.2	2.3 ±0.2	1.9 ±0.05	2.0 ±0.1
Isoleucine	4.6 ± 0.7	5.5 ±0.3	5.4 ±0.43	5.6 ±0.1
Leucine	7.9 ± 1.1*	10.2 ±0.3	7.2 ±0.05*	9.7 ±0.4
Tyrosine	4.2 ± 0.4*	5.4 ±0.3	3.3 ±0.04	4.2 ±0.5
Phenylalanine	3.6 ± 0.6	4.5 ±0.6	3.1 ±0.14*	4.6 ±0.2
Total	50.2 ± 6.3*	67.6 ±3.5	49.2 ±0.3*	66.7 ±2.5
Nonessential				
Serine	9.6 ± 1.3	11.1 ±0.9	8.0 ±1.4	9.6 ±1.4
Glutamine + asparagine	97.2 ± 5.0**	73.3 ±8.4	71.2 ±1.5*	53.9 ±2.3
Proline	14.7 ± 1.0	15.4 ±0.5	18.0 ±4.2	17.1 ±0.6
Glutamic acid	7.3 ± 3.6	4.3 ±1.2	7.1 ±2.4	7.6 ±1.7
Citrulline	3.5 ± 0.5	4.0 ±0.4	4.0 ±0.01	4.3 ±0.4
Glycine	28.8 ± 3.1	23.8 ±1.0	22.2 ±1.1	20.6 ±2.3
Alanine	45.7 ± 4.3**	25.7 ±3.9	45.6 ±3.4	32.5 ±3.9
α-NH_2-*N*-butyric acid	0.7 ± 0.06*	2.6 ±0.8	0.6 ±0.2	1.9 ±0.3
Total	207.6 ±15.4**	160.1 ±9.4	176.7 ±3.8	147.6 ±8.4
E/N ratio [3]	0.24± 0.013**	0.42±0.009	0.28±0.008**	0.45±0.025
No. of determinations	4	5	2	3
Inclusive days of diet administration	40–60	30–55	40–50	30–45

[1] Mean of acidic and neutral amino acids ± SD.
[2] Includes cystine and tyrosine.
[3] For acidic and neutral amino acids.
*, ** Significant difference from values for the 14 g N intake at 1.0% and 0.1% levels, respectively, by *t* test.

TABLE 3

Comparison of nitrogen balance data with selected plasma amino acid values and the essential to nonessential acidic and neutral amino acid ratio (E/N) from subjects receiving various diets

Subject no.	N content of diet	Time diet fed	Plasma amino acids				Total EAAS [2]	Total NEAAS [3]	E/N ratio [4]	Cumulative N balance with given diet
			Valine	Threonine	Glutamine [1]	Alanine				
	g	*days*	*μmole/100 ml*				*μmole/100 ml*			*g*
1	3.5	0	19.2	10.8	51.4	28.6	68	142	0.47	
	3.5	10	13.5	8.8	89.3	34.1	58	187	0.31	−25
	3.5	25	13.2	9.4	103.2	37.5	56	205	0.27	−38
	3.5	35	11.7	6.9	94.5	45.8	48	201	0.24	−43
	3.5	50	12.8	8.3	98.9	48.9	56	225	0.25	−52
	14.0	3	18.1	10.6	81.4	21.1	65	167	0.39	+18
	14.0	10	17.7	10.0	70.9	22.5	63	155	0.41	+38
	14.0	25	18.5	11.4	69.8	22.8	64	131	0.49	+55
3	3.5	0	16.7	9.1	65.9	20.6	59	138	0.42	
	3.5	21	10.0	6.9	90.0	43.3	49	202	0.27	−30
	3.5	30	12.7	7.0	111.1	46.9	50	231	0.22	−34
	14.0	6	19.1	9.5	53.6	24.5	64	145	0.44	+30
6	3.5	0	18.4	13.6	62.2	13.0	65	149	0.44	
	3.5	8	11.6	10.1	69.8	21.9	55	155	0.36	−32
	3.5	25	10.8	9.6	82.3	30.0	52	178	0.29	−57
	3.5	45	11.2	9.2	75.2	36.1	52	178	0.29	−71
	3.5+250 mg valine	8	16.1	8.3	78.1	37.1	56	184	0.30	− 5
	14.0	14	14.0	9.1	63.2	21.3	60	141	0.42	+66
5	3.5	0	17.4	8.3	33.0	25.8	57	116	0.49	
	3.5	15	14.0	8.2	64.8	40.6	54	168	0.32	−21
	3.5	35	11.3	7.0	72.3	43.1	49	174	0.28	−29
	14.0	15	18.8	8.0	46.7	25.6	64	129	0.49	+45
	3.5 (900 kcal)	15	12.9	8.7	64.4	27.7	53	155	0.34	−72
4	3.5	0	22.7	15.4	65.6	35.0	89	171	0.52	
	3.5	14	15.4	11.0	74.8	48.9	68	187	0.36	−36
	3.5	30	12.8	9.7	64.3	55.3	57	181	0.31	−70
	14.0	14	21.4	12.3	58.0	29.0	74	146	0.51	+30
	3.5 (900 kcal)	15	13.5	10.2	68.8	33.3	59	170	0.35	−65
10	0.5	8	12.6	11.0	54.2	46.0	57	184	0.31	−21

[1] Value also represents a small amount of asparagine.
[2] Total essential acidic and neutral amino acids.
[3] Total nonessential acidic and neutral amino acids.
[4] For acidic and neutral amino acids.

Nitrogen balance determinations were carried out with the subjects and in table 3 are shown some comparisons between N loss or gain during the administration of a given diet and plasma amino acid parameters. All subjects continued to lose N throughout the low protein diet period but the variation in amount for the different subjects was considerable. When the low protein diet had been administered for 30 days, the range in N loss was from 25 to 75 g. The rate of N loss was much greater during the first 10 to 15 days and likewise the E/N ratio decreased more rapidly than later in the period. With the exception of subject 4, from 21 to 35 days elapsed before the E/N ratio fell below 0.30 and during this time, from 29 to 57 g of N was lost. This change in the E/N ratio reflected for every subject a decrease in total essential and an increase in total nonessential amino acid values. However, there was considerable variation in the plasma levels of the individual amino acids among the subjects both at the beginning and at the end of the low protein dietary period.

Since the plasma valine value was reduced to such an extent by the low protein diet, a valine supplement of 250 mg/day was given to one subject after he had received the diet containing 3.5 g of N for 45 days (subject 6, table 3). Eight days later, the plasma valine value was elevated to approximately the level observed prior to the administration of the low protein diet, but the other plasma amino acid values and the E/N ratio had not changed appreciably and the N loss continued at about the same rate (0.6 g/day) as in the previous period.

Since the synthesis of nonessential amino acids by body tissues is dependent upon the presence of adequate amounts of the necessary carbon chains, it appeared possible that with a calorie-restricted low protein diet, the increase in nonessential amino acids would not take place. In addition to being given the low protein diet with adequate calories, two subjects, 5 and 4, (table 3) were fed low protein diets containing only 900 kcal/day. After they had ingested this diet for 15 days, their E/N ratio decreased to approximately the same ratio previously obtained for the same time period for the adequate calorie-low protein diet and again the nonessential amino acids were increased. However, as would be expected, much more nitrogen was lost from the body than during the previous trial.

As has been reported (1), alimentation with a high protein diet caused immediate changes in the plasma amino acid levels. When subject 1 received the diet containing 14 g of N for 3 days after a low protein diet period, many of the plasma amino acid values were approaching their pretreatment levels and the E/N ratio had increased to 0.39, although not all of the body nitrogen had been restored. When subject 3 received the 14-g N intake for 6 days following a low protein diet his plasma E/N ratio increased to 0.44 and the nitrogen lost had been regained.

One subject (subject 10, table 3) received a diet of adequate calories, but containing only 0.5 g of N. This very low protein diet was extremely difficult to tolerate for subjects of this age group and no further successful trials were conducted. After the diet had been fed for 8 days, the E/N ratio was low and the valine and threonine values appeared to be reduced, whereas a high value was recorded for alanine. It appears that the removal of all protein from the diet causes essentially the same changes in the plasma amino acid pattern as the diet with 3.5 g of N.

Since, of all the essential amino acids in plasma, valine appeared to be reduced to the greatest extent during trials with the low protein diet, the effect of feeding a diet known to be deficient in valine was studied. Two subjects were fed a mixture of purified amino acids containing the essential amino acids proportioned as in egg protein for one 5-day period and for a subsequent period, a similar diet was fed with valine omitted from the mixture. The results are shown in table 4. For both subjects, the valine value in plasma decreased when valine was removed from the diet. When a plasma sample was taken from subject 4 after one day of eating the valine-deficient diet, the valine was already reduced to its lowest level. In both subjects, the plasma threonine values increased with the valine-deficient diets, an effect not noted with the low protein diets administered previously. With the valine-deficient

TABLE 4

Plasma amino acids values of subjects receiving diets which contained purified amino acids mixtures with and without valine

Amino acid	Subject 11		Subject 12			
	Complete PAA mix [1]	PAA mix [1] without valine	Complete PAA mix [1]	PAA mix [1] without valine		
	Fed 5 days	Fed 5 days	Fed 5 days	Fed 1 day	Fed 2 days	Fed 5 days
	μmole/100 ml	*μmole/100 ml*	*μmole/100 ml*	*μmole/100 ml*		*μmole/100 ml*
Essential [2]						
Threonine	23.9	41.6	14.2	12.0	17.3	19.8
Valine	22.9	12.9	23.6	9.0	11.0	9.1
½ Cystine	12.0	9.8	8.6	8.8	10.1	7.8
Methionine	2.9	3.0	1.9	2.1	2.4	2.4
Isoleucine	7.2	5.7	6.5	4.5	5.4	4.9
Leucine	12.4	11.7	11.8	7.9	9.0	8.3
Tyrosine	6.8	6.6	4.7	3.7	4.2	4.6
Phenylalanine	4.8	5.0	4.1	3.7	4.5	3.9
Lysine	17.4	18.9				
Total	110.3	115.1	75.4	51.7	63.9	60.9
Nonessential						
Serine	15.6	20.5	14.3	11.3	13.0	16.8
Glutamine + asparagine	61.5	73.0	59.3	63.2	74.7	67.3
Proline	15.0	17.2	19.1	14.9	17.0	15.3
Glutamic acid	9.2	3.5	6.5	5.1	8.4	4.2
Citrulline	2.4	3.0	3.7	4.0	2.7	3.6
α-NH_2-*n*-butyric acid	0.9	1.1	1.4	1.3	1.2	1.0
Glycine	36.4	40.3	28.8	32.6	43.2	41.7
Alanine	43.3	47.0	47.7	38.9	44.6	37.8
Ornithine	7.2	6.6				
Histidine	8.5	8.9				
Arginine	4.5	4.2				
Total	204.5	225.5	180.8	171.3	204.8	187.7
E/N ratio [3]	0.54	0.51	0.42	0.30	0.31	0.32
Nitrogen balance, g/day	+0.1	−4.9	−0.2			−5.8

[1] A mixture of purified amino acids proportioned as in egg protein.
[2] Includes cystine and tyrosine.
[3] Molar ratio of essential to nonessential amino acids.

diet the E/N ratio was somewhat lower in only one subject and the nonessential amino acid levels did not appear to be greatly changed.

DISCUSSION

These results have indicated that for men in the age group of 55 years and older, the reduction of the daily protein intake from 14 g of N to 7 g of N did not produce significant alterations in the plasma amino acid pattern, at least under conditions where N balance is maintained. A further reduction of the daily protein intake to 3.5 g of N was associated with a negative N balance and led to marked changes in the plasma amino acid pattern. It has also been observed that when college-age students [4] received the same diet with 3.5 g of N for one week, there were similar alterations in plasma amino acid values.

Since the ingestion of the low protein diet caused nitrogen loss, the question arises as to the precise amino acid deficiencies imposed by the diet. It has already been shown that men in this age group (1) maintain nitrogen balance when 3.5 g of N/day is provided primarily as egg protein, and hence it appears that deficiencies in

[4] Unpublished results.

the low protein diet fed in this study should be related to the essential amino acid content. A comparison of the amino acid composition of this diet with that of egg protein (9) shows that almost all of the essential amino acids in the diet are slightly lower in amount, with the greatest deficiencies being shown for the sulfur-containing amino acids and isoleucine. These amino acids, however, are not those found to be reduced to the greatest extent in the plasma. By contrast, valine, the amino acid which is decreased the most in plasma is not particularly deficient in the diet as judged by comparison with egg protein. In a single subject (subject 6, table 3) to whom a valine supplement was given, although the plasma valine level was increased, the other amino acid parameters and the N balance were not altered as might have been expected if valine were the only limiting dietary amino acid. Subject 10 (table 3) who was given a diet which was practically protein-free also showed a more pronounced decrease in valine than in the other essential amino acids. Arroyave and co-workers (4), measuring plasma amino acids in subjects with kwashiorkor, noted a decrease in valine levels. It appears that when diets low in both quantity and quality of protein are fed over a period of time, reductions occur in the essential amino acids of plasma which may not be directly related to the most limiting amino acid in the diet. It appears possible that factors regulating the metabolic disposal of the various amino acids might exert a predominant influence on plasma amino acids under these conditions, and that regardless of the limiting dietary amino acid, the post-absorption plasma amino acid pattern in protein deficiency states might exhibit common characteristics.

When a diet deficient only in valine was fed (table 4) the plasma valine values decreased within one day. This is in contrast with results obtained with phenylalanine- and lysine-deficient diets (2) which showed that feeding these diets for 5-day periods produced no discernible changes in the plasma levels of the amino acids deficient in the diet. Hence it appears that the low valine values in plasma resulting from the feeding of low protein diets occurred because the plasma level of this amino acid is more immediately responsive to the daily dietary content than are other amino acids. It appears then that in plasma, the valine level is similar to the urea level in that it reflects to some degree the current protein intake. Since the plasma valine level responds so rapidly to a low protein intake, a low plasma valine value might not be indicative of a true protein depletion state unless it occurs in conjunction with decreased levels of other essential amino acids (table 1).

The administration of the valine-deficient diet resulted not only in a reduction in plasma levels of valine (table 4) but in an increase in threonine, particularly in subject 11. This increase in plasma threonine has also been observed with the phenylalanine-deficient diet (2) and as a result of starvation in obese subjects (4) and may be associated with a high rate of protein catabolism.

As stated before (1) and from the results shown in table 1, a low ratio of essential to nonessential amino acids in plasma appears to have promise as an indicator of a protein deficiency state. However, 2 instances have been recorded where a state of protein undernutrition is not associated with a low E/N ratio. The prolonged starvation of severely obese subjects (10) and the administration of purified amino acid diets lacking in phenylalanine or lysine (2) for 5-day periods did not result in decreased plasma E/N ratios. Since neither of these situations would occur under ordinary dietary circumstances, these results do not detract from the potential usefulness of the E/N ratio as an index of protein deficiency. However, the relation of the caloric content of the diet to the plasma amino acid pattern and the E/N ratio should be investigated further. It appears from the studies on starvation that the complete absence of calories prevents the response of the nonessential amino acids. Two subjects in this study who were fed 900 kcal-low protein diets for limited time periods showed increased plasma levels of these amino acids.

There are also situations where the plasma E/N ratio is higher than that reported for the diet containing 14 g of N and these are in severe obesity, in some cases of diabetes and in healthy subjects

eating a ketogenic diet.[5] The higher E/N ratio is caused in all instances by increases in the levels of the branched chain amino acids, and since the various conditions have in common a high gluconeogenesis rate, a high E/N ratio might be indicative of increased gluconeogenesis. Apparently not only the amount of calories, but also the type of calories may influence plasma amino acid patterns.

The amount of nitrogen loss from the tissues was shown to vary greatly for the subjects receiving the low protein diet. It is not known whether this variation resulted from differences in essential amino acid requirements or differences in the labile protein reserves at the beginning of the study. Munro (11) has estimated from various experimental results that 50 or 60 g of N is a fair representation of the labile protein reserve of an adult man, and losses in this range occurred for most of the subjects after they had been fed the low protein diet for approximately 30 days. As this time, none of the subjects had an E/N ratio above 0.31. It is possible therefore that an E/N ratio of less than 0.30 might be taken as an indicator of some degree of protein depletion.

Since this and other studies have shown the plasma amino acid pattern to be influenced by diet, it appears that in various disease conditions where altered plasma amino acid levels are observed, the relationship of these changes with the disease process would be very difficult to establish unless the dietary intake was controlled.

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Rachitogenic Activity of Isolated Soy Protein for Chicks [1]

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ABSTRACT Influence of isolated soybean protein on bone calcification in chicks and the modifying effect of certain dietary substitutions were investigated. Chicks fed a diet containing 40% isolated soybean protein and levels of calcium, phosphorus and vitamin D_3 higher than National Research Council recommendations had reduced bone ash. Supplementation with soybean meal, autoclaving the isolated soybean protein or reducing the level of soy protein improved bone ash. Tibia uptake of ^{45}Ca or ^{32}P was generally increased by soybean meal supplementation but individual variation in uptake was great. A water extract of soybean meal significantly stimulated growth but did not change bone ash. Absorption of ^{45}Ca by intestinal loops of fasted chicks previously fed isolated soy protein was no less than that of chicks previously fed casein-gelatin diets.

Carlson et al. (1, 2) reported that turkey poults became rachitic when fed a semipurified diet containing isolated soybean protein, even though levels of calcium, phosphorus and vitamin D_3 known to be adequate for optimal bone calcification with other diets were used. The rachitogenic effect of isolated soy protein could be partially overcome by increasing the vitamin D_3 level several fold, autoclaving the protein or replacing part of the protein with heated soybean meal. It was suggested that heated soybean meal and other soybean fractions contained an antirachitic factor. Scott et al. (3) showed that availability of anhydrous dicalcium phosphate for turkey poults fed an isolated soybean protein diet was increased by soybean meal and suggested that the meal may contain an unknown factor. An unidentified growth factor in soybean meal for birds has been reported by several workers (4–6).

The present studies were conducted to determine whether the chick would also respond to the rachitogenic effect in isolated soybean protein, to investigate the effect of soybean meal on the tibia uptake of oral doses of radioactive calcium and phosphorus, to attempt to distinguish between the antirachitic effect and growth factor of soybean meal and to study the influence of previous protein source and vitamin D_3 level on radioactive calcium absorption by intestinal loops.

EXPERIMENTAL

The basal diet shown in table 1 was used in all experiments. It contained 1.38% calcium and 0.9% phosphorus by analysis. Chicks were kept in electrically heated, wire-floor batteries in a room from which all outside light was excluded. The fluorescent lamps in the room were covered with polyethylene to reduce to a minimum ultraviolet irradiation of chicks. Two pens of 10 Rhode Island Red male chicks each were used per treatment in experiment 1, 3 pens of 8 White Rock-Cornish crossbred male chicks in experiment 2, and 3 pens of 9 White Leghorn male chicks in experiments 3, 4 and 5. Chicks were given the experimental diets at one day of age in experiment 1, but in subsequent experiments chicks were fed the basal diet for approximately 3 weeks before being given the experimental diets.

Soybean meal used in all experiments was dehulled and contained 50% crude protein. In adding soybean meal or casein to the diets, it was substituted for both isolated soy protein and carbohydrate so that the diets were isonitrogenous. Water extract of soybean meal was prepared by

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[3] Operated by the Tennessee Agricultural Experiment Station for the U. S. Atomic Energy Commission under contract no. AT-40-1-GEN-242.

TABLE 1

Composition of basal diet

	%
Isolated soybean protein [1]	40.0
Glucose monohydrate	50.8
Corn oil	2.0
Dicalcium phosphate	3.3
Calcium carbonate	0.3
Methionine hydroxy analogue	0.5
Glycine	0.3
Minerals [2] and vitamins [3]	2.8

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

[2] Added minerals in mg/kg of diet were: NaCl, 5083; K_2CO_3, 7600; $MgCO_3$, 2080; $ZnCO_3$, 95.9; $MnSO_4 \cdot H_2O$, 153.8; $FeSO_4 \cdot 7H_2O$, 124.5; $CuSO_4 \cdot 5H_2O$, 11.8; $CoCl_2 \cdot 6H_2O$, 2.02; $NaMoO_4 \cdot 2H_2O$, 0.46; and KI, 1.31.

[3] Added vitamins were per kg of diet: vitamin A, 6000 IU; vitamin E, 10 IU; choline, 1.2 g; and (in milligrams) niacin, 50; Ca pantothenate, 24; thiamine, 20; riboflavin, 8; vitamin B_6, 8; menadione, 2; folic acid, 1.2; biotin, 0.24; vitamin B_{12}, 0.01; and ethoxyquin, 250. Vitamin D_3 was added at different levels shown in the tables of results.

stirring one kilogram of meal into 10 liters of tap water at pH 4.7 (acidified with glacial acetic acid) for 20 minutes. The extract was filtered through cheesecloth after which the residue was again extracted under similar conditions. The combined extracts were evaporated to about 50% solids in trays on a steam table. Some of the isolated soybean protein used in experiment 4 was autoclaved at 120° for 30 minutes.

In experiment 1, 4 chicks from each treatment were dosed orally at 4 weeks of age with 20 μc ^{45}Ca (0.8 mg Ca) and 30 μc ^{32}P (0.0003 mg P) in 0.5 ml distilled water /chick and killed 48 hours later for removal of the left tibia. Calcium was separated from phosphorus in the tibia ash by oxalate precipitation and the isotopes were counted with an end-window Geiger-Müller tube with a window thickness of 1.4 mg/cm^2. In experiment 2, 6 chicks from each treatment were dosed with 30 μc ^{32}P in 0.5 ml distilled water/chick and were killed 24 hours later. Bone ash was determined by the AOAC method (7).

A procedure similar to that of Wasserman (8) was used in experiment 6 to estimate ^{45}Ca absorption from intestinal loops. White Leghorn chicks that received the basal diet for one week were divided into 5 groups of 12 chicks and fed diets containing casein with zero, 300 or 3000 ICU vitamin D_3/kg or soy protein with 300 or 3000 ICU/kg. The casein diet consisted of 32% casein and 8% gelatin substituted for the 40% isolated soy protein in the basal diet. Chicks were fasted for 16 hours before start of the in vivo loop experiments so that little or no food was present in the intestinal tract. They were anesthetized by intramuscular injection of sodium pentobarbital (50 mg/kg body weight). A 1-ml solution containing 1 mg Ca (calcium acetate) and 10 μc of ^{45}Ca (0.4 mg Ca) were injected into a ligated intestinal loop. The loop was tied at the yolk stalk and approximately 8 cm anterior to it. Two hours after injection the loop was removed for ashing and determination of the residual ^{45}Ca activity.

RESULTS

Growth rate and bone ash were both significantly increased in experiment 1 by including 30% soybean meal in the basal diet containing 200 ICU vitamin D_3/kg or by increasing the vitamin D_3 level to 2000 ICU/kg (table 2). The response was greater from the higher level of vitamin D_3. Although growth and bone ash averaged higher when soybean meal was added in combination with the higher vitamin D_3, the differences were not statistically significant. Tibia uptake of an oral dose of ^{45}Ca and ^{32}P was markedly increased by the high vitamin D_3 but only slightly by soybean meal. Individual chicks varied considerably in tibia uptake of isotopes as shown by high standard deviations of means.

Chicks receiving soybean meal in experiment 1 were observed to be growing much more rapidly than the other chicks during the last week of the 4-week period. Therefore, in experiment 2, the chicks were fed the basal diet with 300 ICU vitamin D_3/kg for 3 weeks and the experimental diets for only one week. Both levels of soybean meal significantly improved growth at all levels of vitamin D_3 (table 3). Bone ash was also significantly increased by soybean meal except for the 10% level at the lowest vitamin D level. The ^{32}P uptake by tibia was generally increased by soybean meal supplementation, but again individual variation within treatment was considerable.

In view of the results obtained in experiment 2, subsequent experiments were conducted by feeding all chicks the basal

TABLE 2

Effect of soybean meal and vitamin D_3 level on chick growth, bone ash and tibia uptake of radioactive calcium and phosphorus (exp. 1)

Soybean	Vitamin D_3	Avg weight 4½ wks	Tibia ash	Tibia uptake of oral dose	
				^{45}Ca	^{32}P
%	*ICU/kg*	*g*	%	%	%
0	200	222[a] [1]	31.4[a] [1]	2.14 ± 0.53 [2]	2.43 ± 0.51 [2]
0	2000	422[c]	39.9[bc]	4.21 ± 0.37	3.71 ± 0.10
30	200	277[b]	36.0[b]	2.38 ± 0.79	2.60 ± 0.25
30	2000	444[c]	43.9[c]	3.67 ± 0.61	3.33 ± 0.31

[1] Duncan's (11) multiple range test ($P < 0.05\%$). Values followed by the same letters are not significantly different.
[2] SD.

TABLE 3

Effect of soybean meal and vitamin D_3 level on chick gain, bone ash and tibia uptake of radioactive phosphorus (exp. 2)

Soybean meal	Vitamin D_3	Avg gain 7 days	Tibia ash	^{32}P tibia uptake of oral dose
%	*ICU/kg*	*g*	%	%
0	300	94[a] [1]	32.3[a] [1]	2.36 ± 0.44 [2]
10	300	107[b]	33.9[a]	2.98 ± 1.11
30	300	109[b]	37.2[b]	3.25 ± 0.77
0	600	97[a]	34.4[a]	2.26 ± 0.61
10	600	108[b]	37.1[b]	2.86 ± 0.67
30	600	113[b]	39.4[b]	2.92 ± 0.61
0	1200	100[a]	32.5[a]	3.13 ± 0.56
10	1200	121[c]	38.4[b]	3.47 ± 0.62
30	1200	107[b]	39.3[b]	3.36 ± 0.90

[1] Duncan's (11) multiple range test ($P < 0.01\%$). Values followed by the same letters are not significantly different.
[2] SD.

iet for 3 weeks and feeding the experi-ıental diets containing 600 ICU vitamin)$_3$/kg for an 8- to 9-day period. Results f three such experiments showed that ater extract of soybean meal significantly nproved growth but not bone ash (exp. , table 4), autoclaving of isolated soy rotein significantly improved bone ash ut not growth (exp. 4), and addition of asein or simply reducing isolated soy rotein level significantly improved bone sh but not growth rate (exp. 5).

When food material in the intestinal ract was eliminated, the mucosal walls of hicks previously fed isolated soy protein bsorbed as much ^{45}Ca as that of chicks reviously fed casein-gelatin diets (table). The diets influenced bone calcification s shown by lower bone ash of chicks fed he soy diet containing 300 ICU vitamin)$_3$ than that of chicks fed the casein-elatin diet.

DISCUSSION

Although most of the published work on both the soybean meal growth factor and the rachitogenic effect of isolated soybean protein has been carried out with turkey poults, results obtained here demonstrate that the chick will respond in a similar manner. The rachitogenic effect of isolated soybean protein is probably a general phenomenon among monogastric animals as Miller et al. (9) have recently reported that the vitamin D requirement of swine fed soy protein was greatly increased over that of swine receiving a casein diet. Using chicks instead of turkey poults as an assay organism for either the growth factor or rachitogenic effect has the advantage that less total feed and, therefore, less of a test fraction is required. Although uptake of oral doses of radioactive calcium and phosphorus appears to be reduced by isolated soybean

TABLE 4

Response of chicks to soybean meal, water extract, casein and autoclaving or level reduction of isolated soy protein

Diet change [1]	Avg gain	Tibia ash
	g	%
Experiment 3, 9 days		
None	125[a] [2]	37.8[a] [2]
10% Soybean meal	151[b]	38.2[a]
20% Soybean meal	156[b]	40.3[b]
Water extract ≅ 10% soybean meal	146[b]	37.5[a]
Water extract ≅ 20% soybean meal	155[b]	37.4[a]
Experiment 4, 9 days		
None	98[a]	38.1[a]
10% Soybean meal	99[a]	39.2[ab]
30% Soybean meal	112[b]	41.8[b]
Autoclaved isolated soy protein	97[a]	41.8[b]
Autoclaved isolated soy protein + 10% soybean meal	109[a]	42.2[b]
Experiment 5, 8 days		
None	92[abc]	39.1[a]
10% Soybean meal	106[cd]	41.1[b]
20% Soybean meal	112[d]	41.8[b]
10% Casein	98[bcd]	41.6[b]
20% Casein	85[ab]	41.9[b]
Less 10% isolated soy protein	89[abc]	41.6[b]
Less 20% isolated soy protein	74[a]	44.0[c]

[1] All diets contained 600 ICU vitamin D_3/kg.
[2] Duncan's (11) multiple range test ($P < 0.05\%$). Values followed by the same letters are not statistically different.

TABLE 5

Effect of previous diet on radioactive calcium absorption from intestinal loops (exp. 6)

Diet	Vitamin D_3	Tibia ash	^{45}Ca absorbed
	ICU/kg	%	%
Casein	0	35.0[a] [1]	27.8[a] [1]
Casein	300	45.4[c]	43.1[b]
Casein	3000	45.9[c]	46.3[b]
Soy protein	300	37.3[ab]	44.2[b]
Soy protein	3000	42.4[bc]	51.4[b]

[1] Duncan's (11) multiple range test ($P < 0.05\%$). Values followed by the same letters are not significantly different.

protein, the variability in uptake among chicks fed the same diet appears to rule out the use of these isotopes in any routine short-term assay for the rachitogenic effect.

The fact that a water extract of soybean meal significantly increased growth rate, but not percentage bone ash (exp. 3) indicates that the growth factor and antirachitic effect of soybean meal are different. Griffith and Young [4] have also presented evidence that soybean meal contains 2 factors for the turkey poult, a water-soluble factor which improves growth and a factor that remains with the insoluble residue which improves the biological value of phosphorus from anhydrous dicalcium phosphate. Kratzer et al. (10) reported that large amounts of vitamin D_3 did not alter the growth response of turkey poults obtained with a methanol extract of soybean meal, also indicating that factors in soybean affecting growth and calcification were different.

The fact that simply reducing the level of isolated soybean protein in the diet markedly improved bone ash (exp. 5) sug-

[4] Griffith, M., and R. J. Young 1964 Study of fractions of soybean meal responsible for increased growth and phosphorus availability for poults. Poultry Sci., 43: 1323 (abstract).

gests that the "antirachitic" effect of soybean meal may be due to a reduction in level of isolated soy protein. Inclusion of soybean meal in the diet on an isonitrogenous basis required that the level of soybean protein be reduced. The reason that the protein in commercial soybean meal does not reduce bone ash may be that it is heated to destroy animal growth inhibitors. Autoclaving isolated soybean protein reduced its anti-calcification effect for chicks (exp. 4) as it did for turkeys (1).

Unheated isolated soy protein apparently does not interfere with the absorption or metabolism of vitamin D_3 because chicks previously fed isolated soy protein absorbed radioactive calcium as well as similar chicks previously fed casein-gelatin (exp. 6). It appears that isolated soy protein interferes with normal absorption of calcium or phosphorus or of both of these elements.

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