Dietary Composition and Tissue Protein Synthesis

II. TRYPTOPHAN TOXICITY AND AMINO ACID DEFICIENCY IN COLLAGEN SYNTHESIS[1]

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The synthesis of collagen in rats has been shown to vary with age and diet (Nimni and Bavetta, '61b). Thus animals fed a diet deficient in tryptophan showed a marked reduction in the synthesis of skin collagen, while that synthesized around a subcutaneously implanted sponge was only slightly affected by the nature of the diet fed. In addition, it was also observed that these animals had a higher specific activity in their liver proteins subsequent to the injection of a dose of glycine-1-C^{14} than animals fed a diet adequate in tryptophan.

The principal objective of the present experiments was to compare the effects of amino acid excess as well as deficiency on the synthesis of collagen and on the degree of tissue labeling subsequent to the injection of glycine-1-C^{14}. In addition to the studies on the uptake of labeled amino acids by different tissues it seemed pertinent to investigate the activities of enzymes directly involved in the metabolism of amino acids to try to obtain a further understanding of the mechanism of impaired tissue synthesis which results from such dietary changes.

METHODS

Young male rats of the Holtzman strain were used. They were implanted subcutaneously with sterile polyvinyl sponges weighing approximately 100 mg and fed the experimental diets. The composition of the tryptophan-free ration was as follows: acid hydrolyzed casein,[2] 24%; corn starch, 55%; cottonseed oil, 10%; and salt mixture (Hubbell et al., '37), 5%. Adequate vitamin supplementation was provided as previously described (Bavetta et al., '57). Vitamin-free casein[3] was substituted for the hydrolyzed form in the tryptophan-adequate diet. In addition, two other basal diets were used, one with 12% of casein and another with 24% of zein as protein sources. The amounts of tryptophan added to the various diets are shown in table 1.

At periodic intervals, subsequent to the implantation of the sponge, rats were killed and blood and tissue samples taken. Rats were injected intraperitoneally 5 hours before sacrifice with 1 μc of glycine-1-C^{14} (7.3×10^5 count/min; specific activity 4.5 mc/mmole)/100 gm of body weight. Skin, sponge and the tissues under investigation were isolated and protein and nonprotein fractions counted as previously described using a gas-flow ultra thin window Geiger-Muller tube (Nimni and Bavetta, '61a). In addition, the skin and sponge samples containing collagen were gelatinized by autoclaving with water at 15 p.s.i. for 8 hours and dialyzed against water to eliminate radioactivity from the noncollageneous components. Liver arginase was determined by incubating 0.5 ml of a 1% liver homogenate with 1 ml of a solution containing 425 μmoles of L-arginine, 45 μmoles of maleic acid and 20 μmoles of $MnSO_4$, during 15 minutes at 38°C with constant shaking. The pH was adjusted to 9.7 by the addition of buffered arginine. Urea formation was determined on an aliquot by the colorimetric technique of Natelson et al. ('51) using diacetyl rea-

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[2] Salt-free Hydrolyzed Casein, Sheffield Chemical, Division of National Dairy Products Corporation, Norwich, New York.
[3] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 1

Specific activity[1] of collagenous and noncollagenous tissue proteins of rats fed different experimental diets during 14 days

Groups	Diets	Change in body wt[2]	Skin collagen	Sponge		Tissue protein specific activity		
			Specific activity	Total collagen synthesized	Specific activity	Liver	Gluteus	Gastrocnemius
		gm		*mg*				
1	Tryptophan-deficient	−30	10.5 ± 0.7[3]	8.8 ± 0.5	143 ± 18	35 ± 1.9 (11.2)[4]	1.4 ± 0.3 (2.2)	1.3 ± 0.2 (2.1)
2	+0.25% Tryptophan	+63	35.4 ± 1.8	12.4 ± 0.4	134 ± 8	30 ± 2.8 (4.0)	3.4 ± 0.3 (3.6)	4.0 ± 0.2 (3.3)
3	24% Casein	+97	33.1 ± 1.5	12.3 ± 0.5	114 ± 5	28 ± 2.4 (5.2)	4.0 ± 0.1 (2.5)	4.4 ± 0.3 (2.2)
4	+3% Tryptophan	+33	32.3 ± 1.4	11.1 ± 0.7	129 ± 10	24 ± 2.3 (4.8)	3.0 ± 0.2 (4.6)	3.7 ± 0.3 (4.7)
5	12% Casein	+30	30.7 ± 1.4	10.9 ± 0.5	141 ± 9	29 ± 1.7 (3.6)	2.0 ± 0.33 (4.4)	4.0 ± 0.5 (3.5)
6	+1.5% Tryptophan	− 4	24.4 ± 2.7	10.9 ± 0.4	154 ± 18	27 ± 0.9 (4.4)	2.0 ± 0.1 (5.0)	2.5 ± 0.2 (4.7)
7	24% Zein	−26	12.9 ± 0.7	8.6 ± 0.7	174 ± 13	40 ± 1.9 (7.6)	1.3 ± 0.2 (2.0)	1.6 ± 0.2 (2.4)

[1] Count/min/mg tissue protein.
[2] Average initial body weight 130 gm; seven rats/ group.
[3] Standard error.
[4] Figures in parentheses indicate nonprotein fraction radioactivity.

gent. The units represent micromoles of urea formed per milligram of liver. Serum glutamic-oxaloacetic transaminase was determined following the method of Reitman and Frankel ('57). Collagen was determined by a modification of the Neuman and Logan method for hydroxyproline (Bekhor and Bavetta, '61). All values for tissue protein radioactivity are expressed as specific activity (count/min/mg of tissue protein or collagen).

RESULTS

Figure 1 shows the growth retardation induced by the addition of 1 and 3% of tryptophan to a 24% casein diet. The rats consuming a diet supplemented with 3% of tryptophan showed the most marked growth inhibition. Food consumption was influenced by the levels of dietary tryptophan, being lower in those animals consuming the tryptophan-supplemented diets. The uptake of radioglycine by the skin was greatly depressed in animals fed the tryptophan-deficient diet (group 1) or the zein diet (group 7). These results are shown in table 1. A depressed uptake by the skin, although not as marked, was also observed in those animals receiving a diet with 12% of casein supplemented with 1.5% of tryptophan (group 6).

The amount of newly synthesized collagen in the implanted sponge was decreased by the tryptophan deficiency (group 1), 12% casein diet (group 5) and in the animals fed the zein diet (group 7). The specific activity was higher in these latter groups but not significantly so except in the case of the animals fed zein. Table 1 also summarizes the results obtained when the specific activities of liver and skeletal muscle protein and nonprotein fractions were determined. The radioactivity in liver protein and nonprotein fractions showed an increase in rats fed a tryptophan-deficient diet (group 1) as well as those supplied with the incomplete protein zein (group 7).

Since the nature of the diet has been shown to significantly alter the composi-

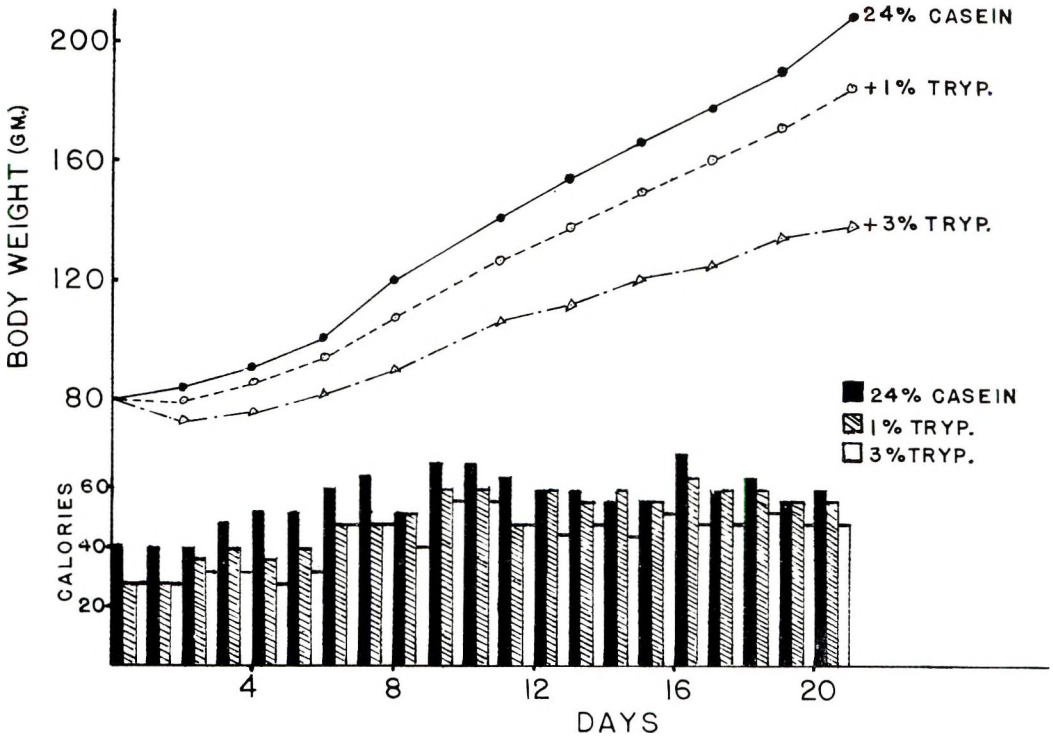

Fig. 1 Growth curve and caloric intake of young male rats receiving different levels of tryptophan supplements in their diets.

TABLE 2

Effect of feeding different experimental diets over a 30-day period on organ weight and skin composition of the rat

Diet	Change in body wt[1]	Liver 100 gm/ body wt	Kidney 100 gm/ body wt	Skin			
				H_2O	Fat	Fat-free solids	Collagen in fat-free solids
	gm	*gm*	*gm*	%	%	%	%
Tryptophan-deficient	−30	3.91	1.00	67.3	3.1	29.6	58.8 ± 1.1[2]
+ Tryptophan, 0.25%	+99	3.76	0.82	65.2	8.9	25.9	52.2 ± 0.93
Casein, 24%	+118	3.87	0.65	64.7	9.2	26.1	53.9 ± 1.2
+ Tryptophan, 3%	+47	4.22	0.96	67.7	5.1	27.2	55.6 ± 1.6
Zein, 24%	−31	4.17	1.00	66.8	3.8	29.4	60.2 ± 1.1
+ Arginine	−33	3.77	0.93	66.5	3.7	29.8	57.4 ± 0.6
Protein-free	−45	3.96	0.88	66.3	3.3	30.4	59.3 ± 1.8
Casein, 6%	−20	4.32	0.92	68.6	4.3	27.1	51.8 ± 0.6

[1] Six rats/group.
[2] Standard error.

tion of skin (Haldi et al., '41–'42) it seemed pertinent to investigate the changes in the proportion of water, fat and collagen that occur during the feeding of the different diets (table 2). The liver and kidney weights were recorded and expressed as a percentage of the total body weight. The diets inadequate for proper growth increased the water, fat-free total solids and collagen content of skin, and greatly reduced the amount of fat. The different types of deficiencies did not appear to exhibit similar effects on the kidney. Kidney weight was relatively lower in those animals receiving the 24% casein diet and appeared to increase with the feeding of imbalanced or hydrolyzed protein diets.

Serum glutamic-oxaloacetic transaminase activity was shown to increase in animals fed a tryptophan-deficient diet, an excess of tryptophan, or low protein (6% casein), as well as in those where zein was the protein component of the diet. This serum enzyme has been reported to increase in activity in cases of liver cell damage (Wroblewsky and La Due, '55). Liver arginase activity was slightly decreased in those animals consuming the low-protein diets and more significantly decreased in the rats fed the 24% zein diet, with or without arginine supplementation, and the protein-free diet. In figure 2 is shown the liver arginase activity plotted against the dietary nitrogen intake.

TABLE 3

Effect of dietary composition on serum glutamic-oxaloacetic transaminase[1]

Diet	Serum glutamic-oxaloacetic transaminase
	μmole units/ml
Tryptophan-deficient	221 (±25)[2]
+ Tryptophan, 0.25%	168 (±11)
Casein, 24%	158 (±15)
+ Tryptophan, 3%	220 (±31)
Casein, 6%	264 (±33)
Zein, 24%	286 (±23)
+ Arginine, 0.75%	285 (±68)
Protein-free	246 (±39)

[1] Six rats/group (experimental period = 21 days).
[2] Standard error.

DISCUSSION

Amino acid deficiency and the toxicity due to excessive dietary tryptophan seemed to exhibit different metabolic effects if measured by their influence on tissue protein labeling, changes in body weight, and tissue composition. Amino acid deficiency completely inhibited growth, whereas the addition of 3% of tryptophan to a 24% casein diet retarded growth to approximately 40% of that of the controls. At a lower level of casein (12%), the addition of 1.5% of tryptophan was able to stop growth completely. In this respect, Sauberlich ('61) recently showed that the addition of 5% of tryptophan to a low-protein diet almost stopped the growth of weanling rats. By increasing the casein level this effect was counteracted emphasizing the

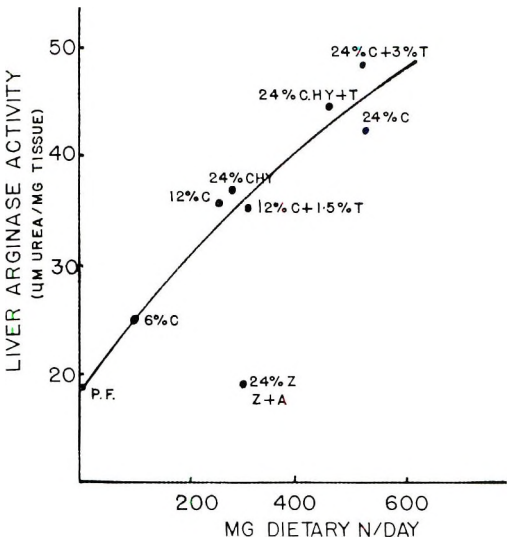

Fig. 2 Liver arginase activity plotted against average N intake during ad libitum feeding of different diets: P. F. (protein-free); 6% C, 12% C and 24% C (6, 12 and 24% casein); 24% CHY (24% casein hydrolysate); T (DL-tryptophan); 24% Z (24% zein); Z + A (24% zein + 0.75% arginine). Each point represents the average of 6 rats adapted to their diets 21 days and killed during absorption.

dependence of the degree of toxicity induced by the excess of an amino acid to its relative rather than absolute proportion in the diet.

The uptake of radioglycine by the skin was decreased considerably in rats fed the amino acid deficient ration. Nevertheless, the percentage of collagen in the dry fat-free skin of these animals shows a relative increase. This does not appear to be the result of increased collagen synthesis but primarily a result of the depletion of the more metabolically labile skin constituents such as fat and noncollagenous proteins. It is therefore to be emphasized that measurements of collagen formation, at a site where previous collagen fibers exist, should be based on radioisotope incorporation rather than on changes in the relative hydroxyproline content.

The uptake of radioglycine may be influenced by the pool of circulating amino acids. The size of this pool undoubtedly is affected by diet as well as by rate of protein synthesis. Thus in the rapidly growing animal, it is conceivable that competition for glycine by other tissues may reduce its availability for synthesis of sponge collagen, whereas more glycine would be available for collagen synthesis in the poorly growing animals. This is seen in the higher specific activity of sponge collagen in the latter group. The reverse situation exists, however, when one compares the protein specific activity of muscles in animals fed a deficient diet as compared with those supplied with adequate diets (table 1).

Liver protein specific activity was increased by the feeding of the amino acid-deficient diets, while the skeletal muscles studied showed a higher rate of labeling in those groups fed the adequate diets.

Radioactivity of the nonprotein fraction of liver was markedly increased in animals fed a tryptophan-deficient diet as well as in those fed the zein diet. However, muscle tissue appeared to behave opposite to that of liver. This difference in behavior appears to be correlated to the degree of catabolic or anabolic activity as evidenced by growth. It also appears as if a consistent effect of the tryptophan-induced toxicity would be the abnormal accumulation of amino acid radioactivity in muscle nonprotein fractions. It is possible that this imbalance at the tissue level could cause an impairment in the proper utilization of the amino acid pool for the purpose of growth. The possibility that this phenomenon may occur during the feeding of other inadequate diets and that this accumulation could be reversed by the addition of specific amino acids should be investigated.

The increased serum transaminase activity in rats fed amino acid-deficient diets and those subjected to a low-protein intake may indicate that some degree of liver cell injury is occurring in these animals. In malnourished babies a nonconsistent increase in serum transaminase was reported by Waterlow ('59). Liver arginase activity appeared to be dependent on the quantity rather than on the quality of the dietary protein ingested. The activity of this enzyme appeared to change in proportion to the amount of consumed nitrogen. A good correlation appeared to occur when these two variables were compared. The only abnormality occurred in

those animals fed the zein diet where the observed values were much lower than those predicted. The addition of 0.75% of arginine to this diet did not influence the low values observed. Although the direct relationship between the levels of dietary protein and liver arginase activity has been well established (Lightbody and Kleinman, '39) the effect of protein quality is not as clear. When 76% of the dietary casein present in a balanced diet was replaced by glycine (Miller, '50) liver arginase activity dropped. In the above experiments the authors indicated a marked toxicity as a result of the excess glycine. The observed decrease in arginase activity may be a manifestation of this toxicity in addition to the usual decreased food intake which accompanies such toxic states. Therefore the latter's claim that the level of arginase does not respond adaptively to variations in dietary nitrogen would probably not hold on the basis of the ingested nitrogen. It seemed more appropriate to relate arginase activity to the actual nitrogen ingested rather than to the amount of nitrogen in the diet. Even more appropriate would be to try and correlate activity with nitrogen absorbed from the intestine. The difference between ingested and absorbed protein would probably explain the unpredicted results observed when zein was fed, a protein that has been shown to be in large proportion excreted in the feces (Geiger et al., '52).

SUMMARY

Skin collagen synthesis as measured by the uptake of radioglycine was considerably decreased in rats fed amino acid-deficient diets. Excess dietary tryptophan (1.5% added to a 12% casein diet) had a similar although less marked effect. The composition of skin with respect to its water, fat, collagen and fat-free total solids was shown to be greatly dependent upon the nature of the diet.

The total amount of collagen synthesized as a response to the subcutaneous implantation of polyvinyl sponges was slightly decreased by the consumption of diets inadequate for optimal growth.

Serum glutamic-oxaloacetic transaminase was increased in the animals fed amino acid-deficient diets, whereas liver arginase activity seemed to reflect the quantity of protein ingested rather than its amino acid composition.

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Carbohydrate and Roughage Requirement of the Cricket, *Acheta domesticus*[1]

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Most insects that require carbohydrates, and many that do not, can utilize sucrose, glucose and fructose very well and a few other sugars to a lesser extent. Pentoses, as a rule, are either very poorly utilized or they are inhibitory to growth and development of most of the insects studied (Gilmour, '61; Spector, '56). Quantitatively, however, one cannot generalize. The carbohydrate requirements of different insects have been shown to vary from no requirement at all for the carpet beetle larva (*Attagenus sp*), or utilization with no requirement for the cigarette beetle larva (*Lasioderma serricorne*) to a dietary requirement of 80 to 85% for the yellow mealworm larva (*Tenebrio molitor*) and the Mediterranean flour moth (*Ephestia Kuehniella*) (Spector, '56).

In preparation for the development of a semi-synthetic (or chemically defined) diet for the cricket, *Acheta domesticus* (L), a study was conducted to determine the qualitative and quantitative carbohydrate and roughage requirement of the insect. Finding a suitable carbohydrate for the proposed semi-synthetic diet presents no problem, for although most of the chemically pure carbohydrates available commercially originate from natural sources, most of them can be synthesized in the laboratory.

Finding a suitable roughage for a semi-synthetic diet was more difficult. For a semi-synthetic diet in the strictest sense of the word, the roughage, as well as all components, should be such that it could be synthesized in the laboratory or defined chemically. The roughage should be non-digestable, metabolically inert, of suitable density so that it would not separate in the diet (nor allow suffocation of the insect attempting to walk on it), nonballing when wet so it will not pack in the stomach of the animal, fibrous to aid in the movement and mixing of diet in the gastrointestinal tract, resistant to swelling in the presence of water, soft and pliable enough to prevent injury to the intestinal lumen, of small enough particle size that it could be eaten by the insect, and free of repelling odors. About the only materials having all of these properties were plastics.

Of the many materials that have been used as bulk or roughage in purified diets, most are cellulose in one form or another. A few examples are oat hull (Hill and Dansky, '54), alfalfa stem meal, psyllium seed husks, ground kapoc, and cellulose (Carlson and Hoelzel, '48; Luckey, '61). A purified cellulose was also used in a previous study of cricket nutrition (Neville, et al., '61) and in part of this study. These materials are not synthetic and it is quite possible that none of these meet the requirement of being metabolically inert. Johnson et al. ('60) have shown that microflora or other actions in the digestive tract enable the rat to utilize a considerable amount of ingested C^{14}-cellulose. It is also well known that the microflora of termites enable them to live on a diet of almost pure cellulose and rumen microbes allow efficient use of cellulose by many herbivores (Stare, '56). Other materials that have been used as roughage or bulk are cellophane, Karaya gum (Carlson and Hoelzel, '48) a poorly defined natural product, and Kaolin (Sibbald et al., '60) a rather dense clay (density of 2.500 gm/ml). Luckey ('61) found cellophane prevented impaction in lambs fed a semi-synthetic diet. Agar-agar and similar materials are used

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experimentally and clinically (Hawk et al., '54).

METHODS

Techniques and environmental conditions previously used for preparing diets, rearing the crickets and analyzing the data were followed (Neville et al., '61). One-day old crickets, *Acheta domesticus* (L), hatched from eggs obtained from a stock colony were used throughout this investigation. Six one-day-old crickets were selected at random and placed in a battery jar (10 × 10 × 10 cm) containing about 2 gm of the respective diet and a cotton stoppered Erlenmeyer flask containing distilled water. Two or three jars were used per diet in each experiment. At the end of the assay period, which was three weeks, the crickets were anesthetized with ether, individually weighed and the data analyzed statistically. The average weight at 21 days of age was the main index of activity used.

Analysis of variance was used to determine the least significant difference (LSD) of the means at the 5 and 1% levels of probability. The LDS obtained from this analysis of variance means that any two weights of crickets within a given experiment having a difference equal to or greater than the LSD are different at the given level of significance. Due to the variability of different batches of insects, all of which were obtained from the same stock colony at varying time intervals, comparisons between average weights of insects were made only within a given experiment. For this reason, the respective LSD's are presented with each experiment.

The compositions of the basal diets were as follows: low roughage (LR) diet, L-arginine, 1.2%; casein, 30%; corn oil, 8%; cornstarch, 30%; cellulose,[2] 12%; sucrose, 12%; vitamin A, 10,000 IU/kg; α-tocopherol, 0.01%; vitamin K_3 (menadione), 0.001%; cholesterol, 0.4%; and B vitamins and total salts as described for the HMC-200 diet (Neville et al., '61). The high roughage (HR) diet was identical to diet LR except that 35% polyethylene[3] (PE) and 19% sucrose were incorporated at the expense of cornstarch and cellulose. Additions and deletions that were made in the LR diet and the sequence relating to the development of the HR diet are given in the result section.

RESULTS

A preliminary experiment was conducted first to determine the effects of diets with and without carbohydrates and roughage. The results, presented in table 1, indicated that during this period in growth the cricket does not require sucrose and starch or glucose as there was no significant difference between the weights of the crickets fed the LR control and those fed the same diet without sucrose and starch or the diets in which glucose replaced sucrose and starch. However, when compared with the control, there was a significant decrease in the weights of those insects fed a diet without cellulose as well as sucrose and starch (group no. 3) or a diet in which glucose replaced all three

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] PE, Alathon "G" polyethylene resin. E. I. duPont de Nemours and Company, Wilmington, Delaware.

TABLE 1

Effects of carbohydrate and roughage on cricket growth

Group no.	Low roughage (LR) diets	Survivors[1]	Avg wt[2]
1	LR control	8	25.8
2	LR minus sucrose and starch[3]	11	18.2
3	LR minus sucrose, starch and cellulose[4]	6	16.1
4	LR with 42% glucose in place of sucrose and starch	9	23.6
5	LR with 54% glucose in place of sucrose, starch and cellulose	10	10.2

[1] Three-week assay: started 12 one-day-old crickets/diet.

[2] LSD 5% = 8.3, 1% = 10.9.

[3] Composition (in per cent) L-arginine, 2; casein, 50; corn oil, 13.3; cellulose, 20; total salts, 13.3; α-tocopherol, 0.17; menadione, 0.0017; B-vitamin mix, 0.7; cholesterol, 0.7; vitamin A, 17,000 IU/kg.

[4] Composition (in per cent) L-arginine, 2.5; casein, 62.5; corn oil, 16.7; total salts, 16.7; α-tocopherol, 0.02; menadione, 0.002; B-vitamin mix, 0.9; cholesterol, 0.83; vitamin A, 21,000 IU/kg.

(group no. 5). The significant difference between the average weights obtained with the 42% glucose diet (containing 12% cellulose) and the 54% glucose diet (12% glucose replacing 12% cellulose) indicated a definite requirement for cellulose or roughage, or both.

The results of table 1, which indicated that carbohydrates were not required by the cricket, did not give evidence that carbohydrates could not be utilized. A qualitative carbohydrate study was conducted with the insect using the low roughage LR diet (table 2). The various sugars replaced the 12% sucrose: all of the diets still contained 30% of cornstarch and 12% of cellulose. Of all the materials tested, chitin, a carbohydrate derivative, was the only one producing a highly significant increase in weight. Lactose and inulin produced weight increases that were only significant between a P value of 0.05 and 0.01. The pentoses, ribose and xylose, and to a lesser extent D-arabinose, caused a significant decrease in the growth rate.

Because replacing sucrose with chitin stimulated cricket growth, it became necessary to see what would happen if chitin were to replace all carbohydrate in the diet. A similar experiment was run on inulin in the event that a higher concentration might prove to be stimulatory. It was also reasoned that if cellulose was needed in the diet simply as bulk or roughage, and not as a source of some unknown growth factor, other inert materials could be substituted for glucose without a reduction in cricket growth. The results of this study are shown in table 3. Apparently chitin and inulin can both be substituted for glucose. However, 30% of chitin caused a significant increase in the growth rate of the cricket, whereas inulin did not (excepting the possibility that 0.1% inulin was stimulatory).

TABLE 2

Qualitative carbohydrate requirement of the cricket with the low roughage (LR) basal diet

Carbohydrate fed at 12% of diet	No. survivors[1]	Avg wt
		mg
LSD 5% 4.8 mg; 1% 6.3 mg		
Sucrose control	10	14.1
Chitin	9	25.7
Lactose	12	19.5
Inulin	11	19.1
Maltose	11	13.8
Glycogen	10	13.0
Trehalose	12	13.0
Dextrin	10	10.6
Cellobiose	11	10.4
LSD 5% 6.1 mg; 1% 8.0 mg		
Sucrose control	11	19.0
D-Mannitol	11	22.3
D-Glucose	12	19.7
D-Fructose	10	18.8
D-Mannose	11	17.9
L-Arabinose	12	17.3
D-Galactose	12	16.0
D-Arabinose	12	12.4
D-Xylose	12	10.3
D-Ribose	10	6.2

[1] Three-week assay: started 12 one-day-old crickets/diet.

The results produced by diets in which cellulose was replaced by other materials (table 3) indicate that the cricket requires roughage for optimum growth. As can be seen, diets containing 12% activated charcoal,[4] agar or silica[5] were comparable to the cellulose controls while the diet with 42% agar was significantly better. Higher concentration of activated charcoal, or silica were also tried but because of the low density of these materials the crickets were unable to survive on diets containing them (the crickets literally drowned in these diets). The most impressive and significant results were obtained with the diets containing the powdered plastics PE and SDB.[6] These diets contained no cellulose and the plastics were added at the expense of starch (the 42% PE and SDB still contained 12% sucrose).

On the basis of the results presented in table 3, growth curves were run comparing cellulose PE and SDB in order to determine the roughage concentration necessary for maximum growth (fig. 1). A particular roughage was added to a 54% sucrose base diet (LR with sucrose replacing starch and cellulose) at the expense of sucrose. The results of these experiments show that cellulose, PE and SDB were equal as roughage and that between 25 and 48% roughage is necessary for "maximum" growth of the cricket. This "maximum" growth rate was

[4] Activated charcoal, Norite-A, Matheson Coleman and Bell, East Rutherford, New Jersey.

[5] Silica, colloidal silica. Godfrey L. Cabot, Inc., Boston.

[6] SDB, styrene-divinylbenzene Copolymer beads, 100-200 mesh, Dow Chemical Company, Midland, Michigan.

TABLE 3

Natural and synthetic sources of roughage

Low roughage (LR) diet changes	No. survivors	Avg wt
		mg
LSD 5% 8.3 mg; 1% 10.9 mg[1]		
12% Cellulose, 42% glucose control	9	23.6
0.1% Chitin, 42% glucose	11	20.9
1% Chitin, 41% glucose	11	23.3
10% Chitin, 32% glucose	10	33.6
30% Chitin, 12% glucose	11	40.1
42% Chitin	11	28.6
0.1% Inulin, 42% glucose	8	32.9
1% Inulin, 41% glucose	10	24.0
10% Inulin, 32% glucose	12	30.2
30% Inulin, 12% glucose	8	27.8
42% Inulin	8	27.6
LSD 5% 7.2 mg; 1% 9.5 mg[1]		
LR Control, 12% cellulose	12	20.1
12% Charcoal,[2] no cellulose	11	27.9
12% Activated charcoal,[3] no cellulose	9	26.2
12% Agar, no cellulose	11	20.4
42% Agar, no cellulose and starch	11	34.0
54% Agar, no cellulose, starch and sucrose	10	17.7
LSD 5% 18.8 mg; 1% 24.8 mg[4]		
LR Control, 12% cellulose	12	30.6
12% PE,[5] no cellulose	12	52.6
25% PE, 17% starch, no cellulose	12	59.7
35% PE, 7% starch, no cellulose	13	90.0
42% PE, no starch and cellulose	13	93.0
12% SDB,[6] no cellulose	12	34.5
25% SDB, 17% starch, no cellulose	14	54.9
35% SDB, 7% starch, no cellulose	0[7]	
42% SDB, no starch and cellulose	12	85.9
12% Silica,[8] no cellulose	6	15.4
12% Molding compound,[9] no cellulose	0	

[1] Twelve crickets started/diet.
[2] Charcoal, bone, Aloe Scientific, St. Louis, Missouri.
[3] Activated charcoal, Norite-A, Matheson Coleman and Bell, East Rutherford, New Jersey.
[4] Fourteen crickets started/diet.
[5] PE, Alathon "G" polyethylene resin, E. I. duPont de Nemours and Company, Wilmington, Delaware.
[6] SDB, styrene-divinyl-benzene Copolymer beads, 100–200 mesh, Dow Chemical Company, Midland, Michigan.
[7] Died from lack of water.
[8] "Cab-O-sil" colloidal silica, Godfrey L. Cabot, Inc., Boston, Massachusetts.
[9] Melamine molding compound, American Cyanamid Company, Bound Brook, New Jersey.

obtained with only 6% of carbohydrate (for diet with the 48% roughage). For future studies 35% of PE and 19% of sucrose were selected as the new high roughage (HR) base diet. In one experiment, the cellulose content was increased to 100%; above 54% the cellulose was used to dilute a mixture of base diet components minus all carbohydrate. Results are also included in figure 1 from a dilution experiment which was prepared by diluting the 54% sucrose base diet with PE. The PE dilution experiment produced but one level of significant growth stimulation. This occurred with the insects fed the 48% PE: 52% basal diet (containing 17.7% of protein). However, the PE dilution diets and the diets containing higher concentrations of cellulose are interesting from the point of view that they show how little nutrients the cricket actually needs to survive and even grow. The 80% PE dilution and the 90% cellulose diets were not significantly different from the 0% roughage

controls even though they contained only 6 and 3% protein, respectively (survival was 64 and 83%, respectively).

A second qualitative carbohydrate study was conducted with the HR base diet as a control (table 4). The various carbohydrate materials were substituted for the 19% sucrose and are the only carbohydrates in these diets. The materials are listed in order of decreasing combined

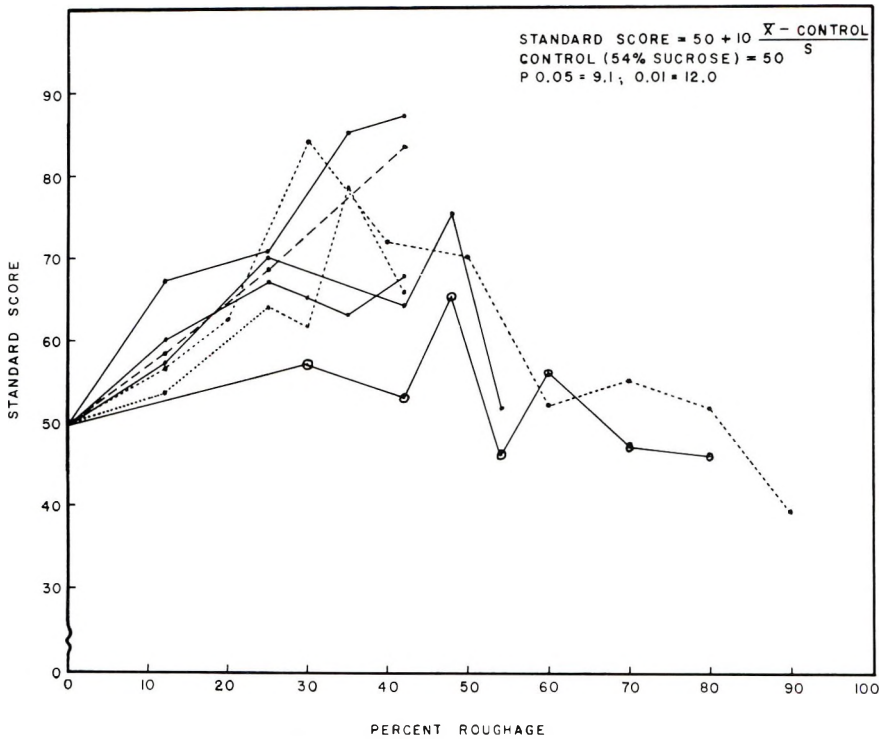

Fig. 1 Roughage growth curves. The low roughage diet containing 54% sucrose (replacing cornstarch and cellulose) was the control. Roughage was added at the expense of sucrose first and then at the expense of a mixture of the remaining nutrients. In calculating the standard score (Dixon and Massey, '57) $\bar{X}$ = mean weight of the crickets fed a test diet, Control = mean weight of crickets fed the control diet and s = the respective standard deviation. Key: . . . . indicates cellulose; - - - -, styrene-divinylbenzene; ——, polyethylene (AG); ○——○, AG dilution diets.

TABLE 4

Qualitative carbohydrate requirement of the cricket with a high roughage (HR) basal diet

Carbohydrate (19%)	No. survivors	Combined avg wt[1]	Carbohydrate (19%)	No. survivors	Combined avg wt[1]
		mg			*mg*
Sucrose	24	53.8	D-Mannose	25	39.4
Trehalose	26	56.6	Corn starch	9	20.3*
Maltose	23	55.1	Inulin	26	19.8*
Glycogen	21	51.8	Chitin	9	16.6*
D-Mannitol	27	49.2	D-Galactose	24	10.7*
D-Fructose	27	48.3	L-Arabinose	24	12.0*
D-Glucose	23	37.6	D-Arabinose	0	0*
Dextrin	16	34.8	D-Ribose	3	9.0*
Cellobiose	23	32.7	D-Xylose	8	8.6*
Lactose	21	31.0	L-Sorbose	10	10.7*

[1] Combined values of two experiments with a total of 30 one-day-old crickets started/diet.
* P values of 0.01 or less.

average weights of crickets so that all of those below lactose produced a significant decrease in the growth rate. Chitin, which was stimulatory in a low-roughage diet, and cornstarch, which had been present in all earlier diets, reduce the growth rate of the cricket significantly when they are the only source of carbohydrates. All of the pentose sugars were found to inhibit cricket growth which is consistent with reports in the literature concerning other insects.

The extent of growth inhibition in the cricket by pentoses is shown in figure 2. In this study, the 19% of sucrose of the HR diet was replaced at various levels by D-ribose, D-xylose or D-arabinose. Four per cent of ribose or xylose (with 15% sucrose) or 8% D-arabinose (with 11% sucrose) caused a significant reduction in the growth rate of the insect.

Since the work of Conrad et al. ('58) and Johnson et al. ('60) indicated the utilization of cellulose in rats, a preliminary study was conducted with rats comparing cellulose and PE as roughage. The results presented in table 5 indicate that PE was an excellent source of roughage for the rat as well as the cricket. The rats showed no signs of objection to or gastrointestinal complications from the PE- or cellulose-containing diets.

DISCUSSION

The results of this carbohydrate study, although they cover only the first three weeks of the crickets' life, are consistent with the reports found in the literature (Trager, '53; Lipke and Fraenkel, '56; House, '61; Gilmour, '61). It appears that carbohydrate is not essential in the diet for survival and growth of the cricket; but that

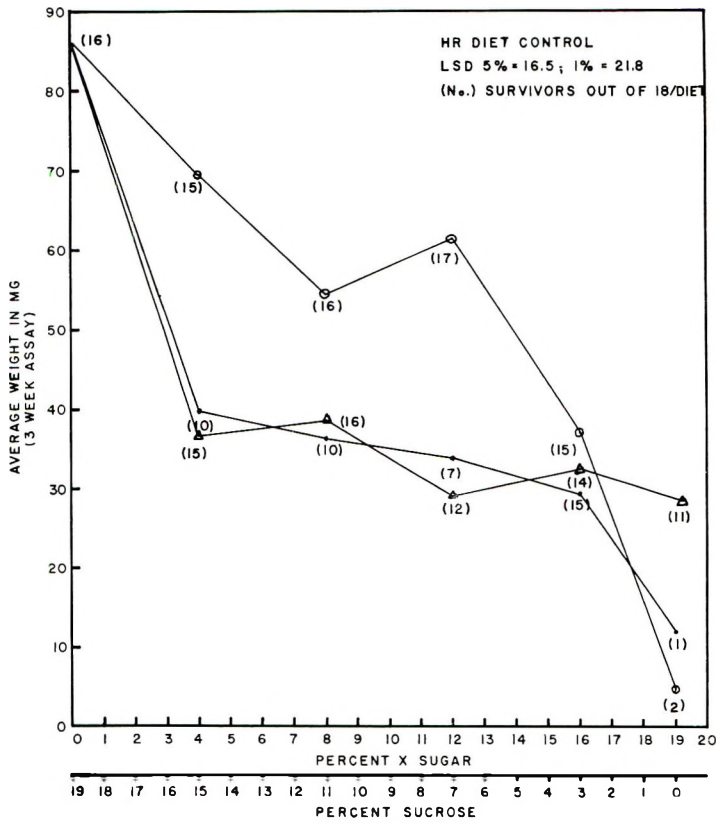

Fig. 2 Pentose: sucrose dilution growth curves. Key: ○——○, D-arabinose; · —— ·, D-ribose; △——△, D-xylose.

TABLE 5

Comparison of rat growth[1] produced by purified diets[2] containing various levels of polyethylene or cellulose as roughage

Roughage	Avg wt gained (cellulose)	Avg efficiency[3]	Avg wt gained	Avg efficiency
%			*mg*	
0	187.8[4]	0.332		
5	209.8	0.343	221.8	0.354
10	203.5	0.327	187.7	0.296
15	176.6	0.302	209.3	0.311
30	187.8	0.274	145.3	0.277
50	80.3	0.151	151.3	0.210
75	−14.3[5]		−13.2[5]	
90	−19.1[5]		−18.2[5]	
Chow diet	219.3	0.341		

[1] Six-week assay using three male weanling rats/diet.

[2] Diet (gm/kg): L-arginine, 10; glycine, 9; L-cystine, 4; casein, 300; glucose (Cerelose, Corn Products Co., Argo, Illinois), 500; choline·HCl, 2; vitamin C, 10; corn oil, 82.72; B-vitamin mix, 2.28 (table 1); total salts, 80 (table 1); fat-soluble vitamins in corn oil (A, 10,000 IU; vitamin D_3, 2000 IU; α-tocopherol, 0.01; menadione, 0.001); roughage at the expense of glucose up to 50% roughage diet, 75 and 90% roughage diets were dilutions of all components except glucose.

[3] Efficiency $= \frac{\text{Weight gained by rat}}{\text{Food consumed}}$

[4] One rat (weighing 235.0 gm) died three days before completion of the assay; its weights and data were used to obtain these values.

[5] Loss of weight of rats that died after being on the diets for one week.

a small amount, as little as 6%, is necessary for maximal growth. In the light of this, the results obtained from feeding pentoses indicate that the growth inhibition produced by the pentose sugars is not due to their being inert but rather to their being toxic metabolically. The fact that 4% of ribose or xylose in the presence of 15% of sucrose (2.66 mmole of pentose to 4.39 mmole of sucrose, or 8.36 mmole of a hexose after hydrolysis during digestion) can significantly inhibit growth is an indication that the toxic effect is more than a simple competition for sites of absorption in the intestine. In the case of D-arabinose, however, competition for absorption sites could explain the result obtained since the ratio of arabinose to sucrose producing inhibition was 8 to 11% or 5.3 to 6.1 mmole (expressed as a monosaccharide). Studies of the actual carbohydrate requirement of the cricket using parameters of longevity and reproduction will be carried out in future experiments with semi-synthetic diets.

The high roughage and low carbohydrate requirements may prove to be very beneficial in developing a semi-synthetic diet for the cricket, since one of the foreseeable problems that could arise with such a diet is too high an osmotic pressure from the use of simple sugars and free amino acids in place of polysaccharides and proteins respectively. A high roughage concentration will not only reduce the amount of sugar and amino acids in the diet but it will also limit the nutrient intake due to the bulk.

Polyethylene or other plastics could possibly eliminate much of the work involved in measuring food intake in larger animals such as rats or chickens. It should be possible to determine the food intake of an animal by gravimetric analysis of the polyethylene found in the feces (from a known concentration of plastic in the diet) since it can be reasonably ascertained that the plastic will not be affected by digestion or by the microflora as is the case with cellulose.

One can only speculate as to the action of roughage in the insect. There could certainly be no common nutrient to be obtained from such diverse substances as polyetheylene, styrene-divinyl-benzene, charcoal, cellulose, chitin and agar. In all probability the effect is mechanical or physical. It is conceivable that roughage could stimulate peristalsis or digestion generally at the same time that it reduces food intake, thereby increasing food efficiency. However, increased food efficiency does not necessarily lead to an increased growth rate. Roughage would increase the

surface area within the intestinal mileux, provide superior emulsification (which can be demonstrated *in vitro*) and probably improve the mixing of nutrients with enzyme by a micro-chopping action.

SUMMARY

An investigation was conducted to determine the approximate quantitative and qualitative carbohydrate and roughage requirements of the cricket, *Acheta domesticus* (L). The insects were fed purified diets for three weeks after hatching, weighed and the data statistically analyzed.

It was shown that although the cricket is capable of utilizing a variety of carbohydrates, a dietary source is not essential for life of the insect during the period of assay; and as is the case with many insects, D-ribose, D-xylose, D- and L-arabinose and L-sorbose are inhibitory to growth.

For maximal growth, roughage is an essential component of the diet. The best growth was obtained with diets composed of 25 to 48% of roughage and 6 to 29% of sugar. Polyetheylene and styrene-divinylbenzene, in a powdered form, were equal to cellulose as a source of roughage for the cricket and polyethylene was equal to cellulose as a roughage for the rat.

ACKNOWLEDGMENT

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Determination of Linoleate Requirement of Swine by a New Method of Estimating Nutritional Requirement[1]

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Biochemical studies of body metabolism are fundamental to nutritional science. Nonetheless, present quantitative definitions of nutritional requirement rest largely upon descriptive statements concerning the presence or absence of "deficiency signs" which are, as yet, not easily definable in biochemical terms. No quantitative procedure to relate nutritional requirements, weight gain, physiological performance changes, or deficiency signs to tissue biochemistry has been described.

In few places is this lack more clearly evident than in studies of essential fatty acid (EFA) nutrition. Here it is evident that a number of polyunsaturated fatty acids (PUFA), either alone or in combination, are capable of overcoming certain EFA deficiency signs. In certain cases the nutritional interactions between these compounds are neither additive nor simple, and suggest a complex pattern of metabolic interrelationships. Any attempt to describe the nutritional requirement for EFA must take into account these underlying metabolic relationships.

Hill and associates ('61) have described the changes that occur in the concentration of PUFA in the tissues of swine maintained with diets deficient in the essential fatty acids. Using the triene/tetraene ratio as an index, the linoleate requirement of swine was estimated, and was shown to be equivalent to that of the rat when expressed as a percentage of the total caloric intake.

A purpose of this paper is to describe an objective procedure for estimating nutritional requirements from tissue analysis data, and to use this process as a basis for discussing the plural nature of the EFA requirement. The data of Hill et al. ('61) are used as a basis for this discussion.

Experimental procedures

The diet composition, experimental plan, and tissue analyses have been described previously by Hill et al. ('61). In those experiments, 66 swine were fed partially purified diets containing known and different amounts of linoleate for periods of time exceeding 56 days. The diet contained casein, skim milk powder, glucose, lard, beef tallow, linoleate concentrate, salt mixtures and the necessary vitamins. A table showing the diet consumed by each animal and the PUFA analysis of heart and liver lipids of each animal has been deposited with the American Documentation Institute, Library of Congress.[3] The PUFA analyses were performed by alkaline isomerization. Subsequent analysis of the fat of each diet by gas chromatography revealed that the linolenate content varied from 0.01 to 0.04% of calories. The composition of each diet is given in the protocol mentioned above.

Equations of curves. Under the conditions of this experiment, tissue PUFA concentrations were dependent upon the amount of linoleic acid in the diet. As seen in figure 1, some types of PUFA in

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[3] Material supplementary to this article has been deposited as Document no. 7264 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document number and by remitting $1.25 for photoprints, or $1.25 for 35 mm microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress.

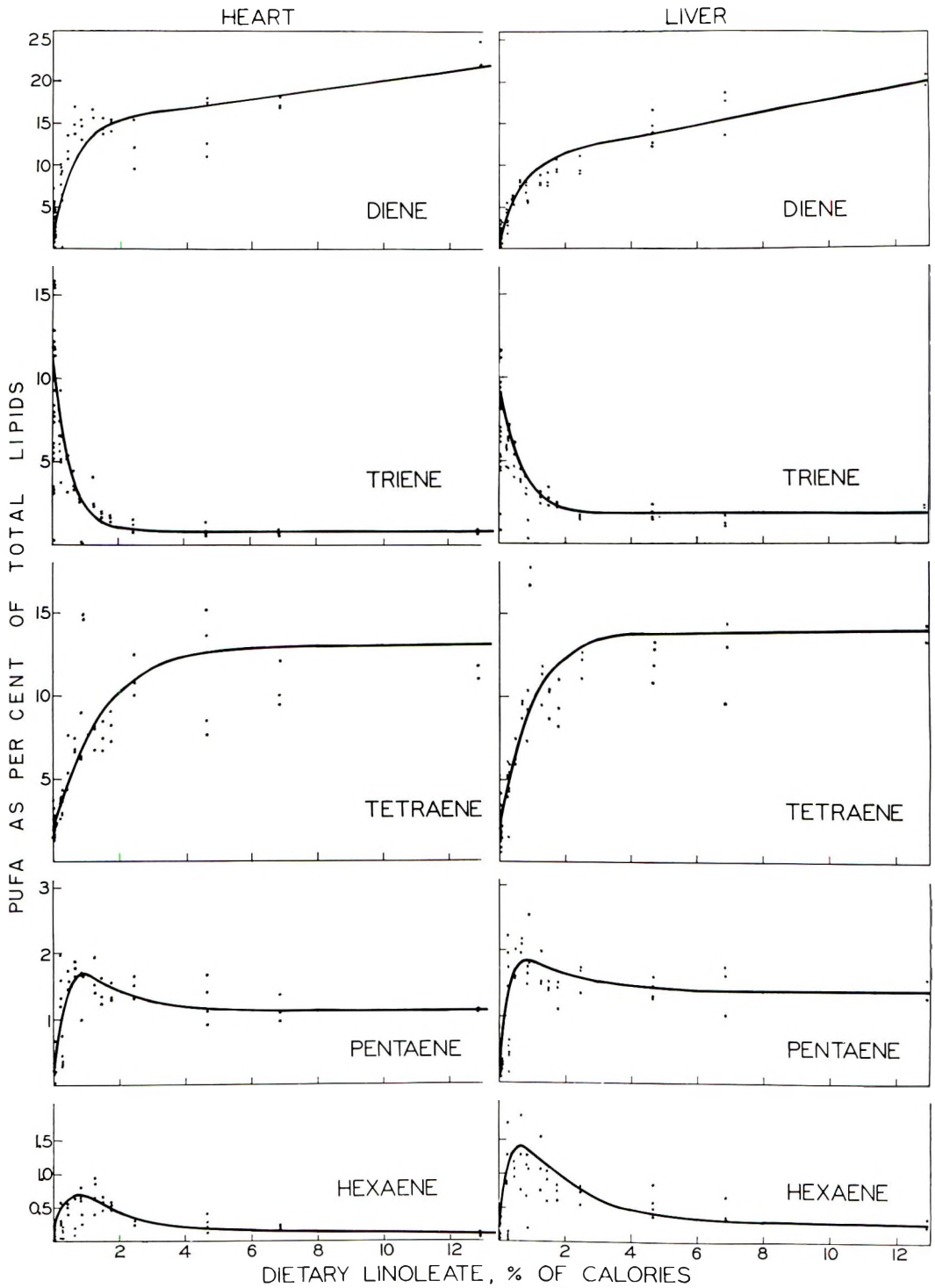

Fig. 1 Relationships between dietary linoleate and tissue PUFA concentrations in the heart and liver of swine. See discussion in text relating to variations in dietary linoleate, which can also influence the shape of the pentaene and hexaene curves.

the heart and liver increased and some decreased as the levels of dietary linoleate were varied from zero to 12.9% of calories. Some appear to undergo several distinct phases of variation. The most rapid changes occur at intakes between zero and 2% of calories, but when one attempts to define the changes for each PUFA component shown in figure 1, they do not begin and end (arrive at final plateau levels) at the same linoleate intakes. Rather, the changes occur at different intake levels and proceed at different rates.

It seems possible that if the descriptive statements of the preceding paragraph could be reduced to a precise mathematical form, the resulting equations might provide a basis for studying the different phases of PUFA metabolism, and for defining the EFA requirement associated with each metabolic phase. As a step in this direction, exponential equations were fitted to the experimental data of Hill et al. ('61). These equations are given in table 1 and are plotted in figure 1 as smooth curves passing through the data.

The advantage of using an exponential equation of this form is that it provides constants that can be reinterpreted in terms of metabolic mechanism and nutritional requirement. The exponent, because it is directly relatable to rate of change, is the term important for this purpose. Tissue PUFA, the variable plotted against dietary linoleate, is taken to represent a steady state level of an intermediate product of lipid metabolism. It is assumed that in a deficiency state the need for EFA is not appreciably altered, and that the decrease in tissue levels of certain fatty acids reflects a decrease in the rate of formation of these products due to a lack of substrate. In the absence of adequate amounts of normal substrate, certain other fatty acids may enter competitively into an enzymatic pathway to give rise to products which will accumulate abnormally in the tissue. For example, by a series of dehydrogenations and a 2-carbon addition reaction, linoleate is normally converted to arachidonate. When linoleate, the normal substrate, is lacking, oleate may be converted (Mead and Howton, '57) to 5, 8, 11-eicosatrieneoate (Mead and Slaton, '56). As studied by the alkaline isomerization procedure, this process, under conditions of linoleate deficiency, would give rise to an increase in trienoic acid with a decrease in tetraenoic acid levels in the tissues of an animal deficient in a dienoic acid. Actually, alkaline isomerization may measure several compounds in each unsaturation group. Some of these compounds may be decreasing while others are increasing or undergoing little change (Klenk and Oette, '59).

TABLE 1

Equations[1] of curves in figure 1

Heart	
Diene	$Y = 14(1-e^{-2x})+24(1-e^{-0.03x})$
Triene	$Y = 0.8+11\,e^{-2x}$
Tetraene	$Y = 2+11(1-e^{-0.7x})$
Pentaene	$Y = 1.1+1.3\,e^{-0.7x}-2.2\,e^{-3.4x}$
Hexaene	$Y = 0.12+1.08e^{-0.6x}-e^{-3x}$
Liver	
Diene	$Y = 9(1-e^{-2x})+18(1-e^{-0.07x})$
Triene	$Y = 1.8+7e^{-1.7x}$
Tetraene	$Y = 2+12(1-e^{-x})$
Pentaene	$Y = 1.1+1.4\,e^{-0.7x}-2.2\,e^{-3.4x}$
Hexaene	$Y = 0.3+2.0\,e^{-0.6x}-2.1\,e^{-3.4x}$

[1] In each case X represents dietary linoleate expressed as a percentage of the total calories, and Y represents the PUFA concentration in tissue lipids specified in the left column. These equations were derived for the experimental conditions in this investigation and should not be generally applied to other species or different conditions. The form of the equations for pentaene and hexaene is discussed in the text. Their shape may have been influenced by the small amounts of dietary linolenate. Dietary variations in linolenate which may have influenced the shape of the pentaene and hexaene curves are discussed in the text.

To relate these various possibilities to the equation in table 1 it is helpful to think in terms of the following models.

Model 1

Assume that Y represents the measured tissue concentrations of some polyene, y, which is derived from substance, a, by a metabolic processes

$$a \xrightarrow{k} y$$

and assume that the rate, r, of the formation of y is a function of both a reaction rate constant, k, and the substrate concentration, A. A decreased rate of formation of y might then be expected in cases of dietary restriction if there is a lack of substrate, a, or if the rate of the metabolic process is decreased due to a deficiency of some vitamin cofactor. In the present instance the substrate concentration is

the effective variable, x, and the rate of formation of y is directly dependent upon dietary linoleate intake. If there is a constant demand for y, the data for the tissue concentration of this component could be described at various levels of linoleate intake by a curve of the type:

$$Y = Y_n(1 - e^{-rx}) \quad (1)$$

where Y_n is the "normal" tissue polyene concentration oberved at linoleate intakes in excess of the minimum requirement, r is a constant, and e is the base of the natural logarithm.

Model 2

By one of several possible mechanisms, Y may increase with a decreasing linoleate intake after the fashion:

$$Y = Y_0 e^{-rx} \quad (2)$$

where Y_0 is the tissue concentration at a zero linoleate intake. Such a change could result if the blocking of a main pathway ($a \rightarrow b$) led to the formation of a secondary product, y:

$$a \xrightarrow{k} b$$
$$\searrow$$
$$y$$

or by the interaction between a pair of related reactions such as: linoleate → arachidonate, and oleate → eicosatrieneoate, as discussed above. For simplicity, the model reactions, and equations (1) and (2), assume that Y, the measured tissue concentration, represents a single chemical compound, y, whereas, in the present study, the alkaline isomerization data represent measurements of entire classes of PUFA (i.e., the sum of all dienes, trienes, etc.). Hence it is not unexpected that some of the equations of table 1 are of the form:

$$Y = ae^{-r_1x} + be^{-r_2x} \quad (3)$$

This can be interpreted as indicating that in these cases the observed tissue concentration changes represent the resultant of two or more distinct processes and may involve more than one chemically distinct compound. In such instances, both the fitting of the curves and the interpretation of the results should be approached with reservations. This point will be considered further in the discussion of the estimated linoleate requirement of swine.

Basis for computing dietary requirements. Classically, the minimum dietary requirement of any nutrient has been defined as that amount that will prevent the development of any of the signs of dietary deficiency. Conversely an intake that does not allow a maximum growth rate, or at which one can demonstrate metabolic changes indicative of the deficiency condition, is defined as an intake below the minimal requirement for that nutrient. The problem then becomes one of detecting a difference between maximal performance and the performance observed in a group of animals at some specific intake level. The smallest significant change in response that can be measured is dependent upon the precision of the experimental method and the variation between animals.

In the present study, a different approach was taken to the problem of determining the relationship between intake and metabolism. Exponential (first order) curves have been fitted to the experimental data. By their very nature there is no "breaking point" in these curves. There are continuous although extremely small changes in these curves even at the highest linoleate intakes. The most characteristic feature of a first order reaction curve is the half-life period which it shows. Since we take the abscissa in this case to be represented by linoleate intake rather than by time, it is appropriate to term this a "half-change" intake value. We will represent this half-change intake with the abbreviation $I_{1/2}$ and compute it from the r-values in the equations of table 1 by means of the relationship:[4]

$$I_{1/2} = \frac{\ln 2}{r} = \frac{0.693}{r} \quad (4)$$

We define $I_{1/2}$ as the nutrient intake which provides a biochemical or physiological response lying midway between that found at a zero intake and that found at an adequate intake of the critical nutrient.

[4] The derivation of the parent relationship equating half-life to velocity constant can be found in Glasstone, Textbook of Physical Chemistry, D. Van Nostrand Company, Inc., New York, 1940, p. 1027 or in any standard physical chemistry text. The parent equation has been useful in describing monomolecular reactions such as the decay of a radioactive isotope, and pseudo monomolecular reactions as are found in metabolic situations. In these cases a half-life time is described. Here a parallel phenomenon, a half-response intake is involved.

An estimation of minimum requirement by classical methods requires the ability to distinguish between effects observed in the slowly changing region of the intake-response curve near the point at which the response is asymptotically approaching a maximum level. Small errors in chemical and physiological measurement can lead to large errors in requirement estimated under these conditions. From the statistical considerations involved, the greater the experimental uncertainties, the lower the resulting requirement estimate. By contrast the $I_{1/2}$ value is defined and measured in a rapidly changing portion of the intake-response curve. Experimental variability may lead to either positive or negative differences in $I_{1/2}$ estimate, but the effect of these errors will be small, i.e., the error/slope ratio will be minimal in the rapidly rising part of the curve.

It is a characteristic of a simple first-order curve that the abscissa of any point on it can be defined in terms of $I_{1/2}$ units. Hence, whatever point is chosen to represent the minimum nutritional requirement (MNR) it follows that:

$$MNR = n\ I_{1/2} \qquad (5)$$

where n is some constant of proportionality that remains to be determined. We assume this constant to be approximately 1.7, which yields the relationship:

$$MNR = 1.7\ I_{1/2} = \frac{1.2}{r} \qquad (6)$$

The assumption that n lies close to 1.7 is based upon the two following considerations:

(1) Values of n in the range 1.8 to 2.3 yield overall MNR estimates that are reasonably consistent with previous judgements concerning these same swine data (Hill et al., '61) and with MNR estimates from similar data relating to the rat (Holman, '60; Caster and Holman, '61).

(2) Data from quite different nutritional studies are consistent with values close to n = 1.5. For example, if one considers the changes in pyramin excretion at different thiamine intakes as reported by Mickelsen et al. ('47) it is found that $I_{1/2}$ = 1.25 mg of thiamine/day. Considering the changes[5] in plasma pyruvate under dietary stress conditions as reported by Caster ('57), it is found that $I_{1/2}$ = 0.85 mg of thiamine/day. In view of the small numbers of data involved, it is doubtful if there is any real difference between these two values. The NRC ('58) thiamine requirement for this group of young males on approximately a 3,000 Cal/day diet is 1.5 mg of thiamine/day. This yields values of n = 1.2 and 1.8, respectively, for these two cases. In like manner, the white cell vitamin C data of Krebs ('53) yield estimates of $I_{1/2}$ = 50 mg of vitamin C/day. From the NRC requirement of 75 mg/day, it can be estimated that n = 1.5. Because of the uncertainties associated with the built-in safety factors inherent in all present requirement estimates, it is doubtful whether any precise and universally acceptable value for n can be determined in the near future. The minimum nutritional requirement (MNR) units are the most familiar of the terms used here, and thus will tend to be used. It must be remembered, however, that r and $I_{1/2}$ values are the more fundamental data and thus should always be reported as a basis for possible later reevaluation.

Linoleate requirement of swine. Figure 2 summarizes the frequency with which coefficients (r-values) of different magnitudes appeared in the equations of table 1. Along the upper edge of this figure are indicated the corresponding MNR values and $I_{1/2}$ values as computed with equation (6). Those squares with a cross-hatched background are derived from the liver data. The others are from heart. In addition, figure 2 includes one r-value obtained from the equation:

$$Y = 295\ (1 - e^{-x}) \qquad (7)$$

which relates y, weight gain in grams per day, to x, dietary intake of linoleate. This square is marked with W.

In figure 2 there appear to be 4 distinct groups of r-values corresponding to MNR values of 0.4, 0.7, 1.5 and 20 Cal. of linoleate/100 Cal. of diet, respectively. The first of these groupings (MNR = 0.4, $I_{1/2}$ = 0.2 and r = 3.3) relates to pentaene and hexaene metabolism. This was unexpected. Current biochemical concepts (Klenk and Mohrhauer, '60; Widmar and Holman, '50) tend to relate tissue pentaene and hexaene metabolism with dietary

[5] Starting with the data in the right column of plasma pyruvate data in table 2 of this reference, plot the differences above a normal pyruvate level of 0.9 mg/100 ml against the long-term thiamine intakes.

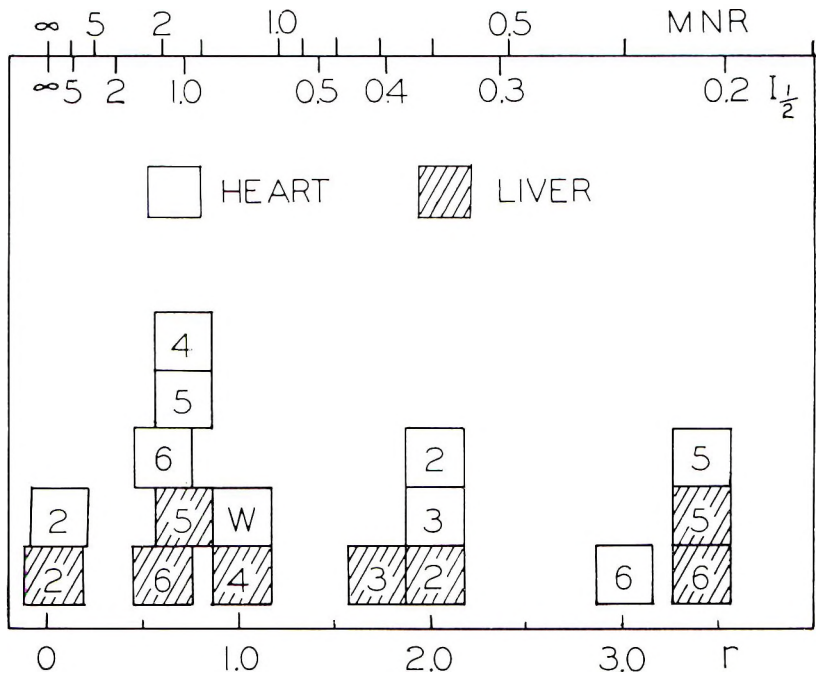

Fig. 2 Linoleate requirement estimates derived from the r-values of the equations in table 1. The r-value in each case is the coefficient of x in the exponent of e. The numerals in the squares indicate the number of double bonds in the tissue polyenes measured by alkaline isomerization. The W is an r-value derived from weight data. Minimum nutritional requirement (MNR) is related to $I_{1/2}$ by equation 6 in the text.

linolenate rather than with dietary linoleate. There were variations in linolenate intake in this experiment ranging from < 0.01% (diets 5, 7, 16 and 17) to 0.04% (diets 18 and 19) of calories. These variations in dietary linolenate are small, but under the circumstances may not be completely negligible. For the present, therefore, we refrain from drawing conclusions concerning the nutritional significance of this group of r-values, and suggest that in further studies particular attention be paid to possible linoleate and linolenate interrelationships at low intake levels which may be related to tissue pentaene and hexaene metabolism.

In figure 2 it is seen that the second group (MNR = 0.6; $I_{1/2}$ = 0.3; r = 1.9) is related to tissue diene and triene metabolism while the third group (MNR = 1.5; $I_{1/2}$ = 0.8; r = 0.8) is related to tissue tetraene, pentaene and hexaene metabolism. Because of the reasons stated above, we have reservations about interpreting the pentaene and hexaene data of this experiment in terms of nutritional requirements or metabolic relationships. It is, perhaps, significant that the body weight gain data yield an r-value in the range of the third group. This would suggest that all of these three metabolic requirements must be satisfied before one could expect a maximum response from such a general indicator of body well-being as weight gain.

There is a fourth group (MNR = 20; $I_{1/2}$ = 12; r = 0.05) in figure 2 which is related to changes in tissue diene concentrations. We interpret this as being related to the storage of linoleate in depot fat under conditions of dietary excess, and is not meaningful in terms of a nutritional requirement for EFA.

We do not specify 1.5% of calories as being the minimum dietary requirement for linoleate, but rather think of it as the amount of linoleate needed to satisfy all three of the EFA metabolic requirements under the conditions of the present experiment. If arachidonate or some other polyunsaturated fatty acid can satisfy

more efficiently one or more of the higher requirement levels, the pure linoleate requirement could be correspondingly smaller. Experimental work is now in progress to test this possibility.

DISCUSSION

Hill et al. ('61) have indicated that the linoleate requirement of swine is equal to that of the rat when expressed as a percentage of the caloric intake. If this is true, it is perhaps pertinent to consider the tissue analysis results for the rat (Caster and Holman, '61) together with the present swine data, and review both of these sets of data in terms of the changes observed at specific ranges of linoleate intake.

From factor analysis studies it is evident that the major changes in tissue composition occur in the rat as the linoleate intake is increased from zero to 0.14% of calories (Caster and Holman, '61). In the intermediate intake range, 0.14 to 0.5 Cal./100 Cal. of diet, there are additional changes which seem to be influenced by the total lipid content of the diet. There is very little change above intakes of 1.0 Cal. of linoleate/100 Cal. of diet. It is true, however, that an examination of the tissue analysis data for these rats reveals small but significant ($P < 0.01$) differences in diene, trienes, etc. occurring between groups fed 4% and those fed 20% of their calories as linoleate. There is a tendency to discount the nutritional significance of these small differences, however, when the total data are examined and the effects of changes in total fat intake are noted.

Hill et al. ('61) showed that a similar situation obtained in swine nutrition. There are evidences of change at linoleate intakes ranging from 0.2 to 2% of calories. Protection against aortic lesions was provided by intakes of 0.2 to 0.3% of calories. The intersection point in the triene/tetraene ratio plot occurred at 0.3 to 0.4% of calories but a final plateau value is not reached by this index until intakes of 2% of calories are ingested.

All of these observations, then, are in agreement with the view that linoleate is satisfying not one but several metabolically distinct EFA requirements. In the past, the tendency has been to center major interest in determining what level of linoleate would satisfy the total EFA requirement. The data of figure 2 suggest that a substantial portion of the metabolically useful information from feeding and tissue analysis experiments is to be obtained at dietary intakes which are three- to tenfold below this level.

The historic purpose for measuring a nutritional requirement is related to the prevention or cure of symptoms associated with a deficiency state. In the present study, this relationship is not direct and obvious. EFA deficiency was originally observed in rats and these observations were extended to several other species. The dermatitis observed in rats, mice, dogs, guinea pigs, cattle, insects and humans is not easily observed in swine; and no dermal symptoms appeared in the animals used in this study. Nevertheless, the biochemical aberrations in the PUFA pattern were amply demonstrated to be related to dermal symptoms in several species, and it is this biochemical lesion that concerns us here and which makes assessment of EFA status in swine possible. Moreover, weight reponses in rats and in swine have been found to parallel linoleate intake and severity of the biochemical lesions. Thus, the PUFA pattern, which can be measured with more precision than dermatitis, appears to us to be the better index of EFA deficiency.

SUMMARY

A new method of estimating minimum nutrient requirements is proposed which is particularly well suited to the interpretation of tissue analysis or weight gain data. For the first step of this procedure, exponential equations were fitted to the curves relating tissue PUFA concentration to dietary linoleate concentration. Constants derived from these equations were then used to demonstrate that there are 4 distinct metabolic processes related to linoleate nutrition. Two of these were found to correspond to linoleate requirements of 0.6 and 1.5% of calories, respectively. The former of these (0.6 Cal./100 Cal. of diet) was associated with the decrease in tissue diene and increase in tissue triene that is observed as linoleate intake decreases. The latter (1.5 Cal./100

Cal. of diet) was associated with tetraene metabolism in the tissues, and total body weight gain. The other two metabolic processes were not interpreted in terms of nutritional requirements. One was related to the storage of excesses. The other was associated with tissue lipid changes observed at linoleate intakes below 0.4% of calories, and might be referable to variations of linolenate in the diets used in this experiment. These latter changes appear complex and require further study before interpretation.

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Studies on the Availability of Calcium Orthophosphates to Chickens and Turkeys

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The comparative biological utilization of different phosphatic materials has been the subject of several studies. Since numerical ratings of such compounds are relative rather than absolute, some standard reference must be selected and other compounds compared with the standard. Some confusion exists in the literature because investigators have not always recognized (1) that many phosphatic compounds may exist in several states of hydration and crystal modification and (2) that different species such as the chick and the turkey may differ significantly in their ability to digest and metabolize the same phosphatic compound.

In some of the first work on this subject, Gillis and associates ('48) reported on the utilization by the chick of phosphorus from various sources, including *ortho-*, *meta-* and *pyro-* forms of some calcium, sodium and potassium salts of various physical forms. However, in this study the phosphate compounds were fed at levels too high to make an accurate evaluation of the chick's ability to utilize the more available compounds.

In later, more critical studies, Gillis et al., ('54) reported comparative values for the utilization of various phosphate materials as compared with β-tricalcium phosphate which was arbitrarily assigned a value of 100. They reported values for dicalcium phosphate (reagent grade) and monocalcium phosphate (reagent grade) of 98 and 113 respectively. Wilcox and coworkers ('54) reported the availablity of phosphorus from different sources for turkey poults. They found that the phosphorus in monocalcium phosphate was well utilized by the poult and that the phosphorus from dicalcium phosphate (USP grade) was utilized to a lesser extent, while the phosphorus in tricalcium phosphate (NF grade) and β-tricalcium phosphate was poorly utilized by the turkey poults as compared with the other calcium orthophosphates. Scott et al. ('56, '62) observed that the phosphorus in anhydrous dicalcium phosphate (reagent grade) was poorly utilized by the poult.

It was shown by Gillis et al. ('48) that the solubility of phosphates in weak acid is not a reliable criterion for estimating the availability of phosphates to animals. Such tests as the solubility in neutral ammonium citrate and weak acids were originally designed as a rough approximation of the availability of phosphatic fertilizers to plants. Even in this application the correlation is poor. However, there is a need for further study on the relationship between citrate solubility and utilization of phosphates by animals.

The studies reported in this paper compared the solubility and biological values of various types and chemical grades of calcium phosphate. Chicks and turkey poults were the experimental animals.

EXPERIMENTAL

Chemical and solubility determination. The volumetric method described in AOAC ('55) was used to determine phosphorus in the various samples and soluble fractions. The water-soluble and the citrate-soluble phosphate fractions were also determined by the procedures described in AOAC ('55).

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Phosphate samples

The source and, when known, the method of preparation of the calcium phosphate samples used in this investigation are described. The chemical data obtained on these samples are presented in table 1.

Sample no. 1. β-Tricalcium phosphate, β-$Ca_3(PO_4)_2$. This sample was prepared in our laboratory.[3] A mixture of 10,000 gm of conditioner grade "tricalcium phosphate" (contained by analysis, calcium 37.1%, and phosphorus 17.7%) and 1,750 gm of food-grade dicalcium phosphate (calcium 29.9% and phosphorus 23.4%) were digested with continuous agitation in water kept near the boiling point for two days, to obtain a uniform mixture. The product was washed in acetone and dried in a steam oven. It was then ignited by raising the temperature to 1,000°C for about one hour. X-ray and chemical analyses of this material showed it to be β-tricalcium phosphate.

Sample no. 2. Tricalcium phosphate (NF), $Ca_3(PO_4)_2$. This material is a precipitated calcium phosphate and meets the specifications outlined in the National Formulary ('55). This material is "a variable mixture of calcium phosphate having the approximate composition $Ca_{10}(OH)_2(PO_4)_6$. After ignition at about 800°C for 30 minutes, it contains an amount of phosphate (PO_4) equivalent to not less than 90% of tri-basic calcium phosphate, $Ca_3(PO_4)_2$."

Sample no. 3. Tricalcium phosphate (AR), $Ca_3(PO_4)_2$. This was the analytical reagent (AR) grade of tricalcium phosphate purchased from a large chemical supply house.

Sample no. 4. Dicalcium phosphate (USP), $CaHPO_4 \cdot 2H_2O$. The preparation, properties and specifications of this material are outlined in the Pharmacopoeia of the United States ('55). It is described as "dibasic calcium phosphate, contains not less than 98% of $CaHPO_4 \cdot 2H_2O$ calculated on the anhydrous basis." Upon ignition to constant weight at 800–825°, it should lose not less than 24.5% and not more than 26.5% of its weight.

Sample no. 5. Dicalcium phosphate (AR), $CaHPO_4$. This is the analytical reagent grade of dicalcium phosphate and is the anhydrous salt. Theoretically, when ignited at 900°C to a constant weight, $CaHPO_4$ (monetite) should lose 6.62% of weight. This is in contrast to $CaHPO_4 \cdot 2H_2O$ (brushite) which should lose 26.17% of its weight. The loss in weight on ignition is caused by a loss of water and conversion to calcium pyrophosphate ($Ca_2P_2O_7$) for both the $CaHPO_4 \cdot 2H_2O$ and $CaHPO_4$. Samples 4 and 5 did not vary greatly in their solubility in neutral ammonium citrate; however, the dihydrate form appeared to be slightly more soluble in water (table 1).

Sample no. 6. Monocalcium phosphate (AR), $Ca(H_2PO_4)_2 \cdot H_2O$. This is the analytical reagent grade monocalcium phosphate. It is 100% soluble in water.

Sample no. 7. Dicalcium phosphate, $CaHPO_4 \cdot 2H_2O$. This sample was prepared in our laboratory by the method outlined in Inorganic Syntheses ('53). According to the reaction $Na_2HPO_4 \cdot 2H_2O + CaCl_2 \cdot 6H_2O \rightarrow CaHPO_4 \cdot 2H_2O + 2NaCl + 6H_2O$. The reaction was conducted at room temperature at pH 4 to 5, using analytical reagent grade chemicals and distilled water. The air-dried product lost 26.25% of its weight upon ignition against a theoretical loss of 26.17%.

Sample no. 8. Dicalcium phosphate, $CaHPO_4$. This sample was also prepared in our laboratory by the method outlined in Inorganic Synthesis ('53). The procedure was similar to that used in the preparation of $CaHPO_4 \cdot 2H_2O$, except that the reaction temperature was maintained at 100°C, and the wash solutions differed slightly. The product lost 6.94% of its weight upon ignition against a theoretical loss of 6.62%.

Samples no. 9, 10, 11 and 12 were commercial samples of dicalcium phosphate that were collected in the laboratory to determine whether their structure was as indicated by their manufacturing labels. The weight loss on ignition of these samples showed the analytical reagent grade samples to be anhydrous dicalcium phosphate. However, one of the USP samples tested (sample no. 9) was apparently not $CaHPO_4 \cdot 2H_2O$, as labeled (table 1). This

[3] The authors express their appreciation to Mr. W. L. Hill of the U.S.D.A., Beltsville, Maryland, for information on how to prepare the material.

TABLE 1

Analytical and solubility data on various calcium phosphate samples

Sample no.	Name	Chemical formula	Ca	P	Citrate-soluble phosphorus	Water-soluble phosphorus	Weight loss on ignition[1]
			%	%	%	%	%
1	β-Tricalcium phosphate	β-$Ca_3(PO_4)_2$	39.4	19.5	8.9	0.01	
2	Tricalcium phosphate NF	$Ca_3(PO_4)_2$	36.6	16.9	6.3	0.02	
3	Tricalcium phosphate AR	$Ca_3(PO_4)_2$	37.5	18.7	4.5	0.15	
4	Dicalcium phosphate USP	$CaHPO_4 \cdot 2H_2O$	23.7	18.4	17.8	0.50	25.2
5	Dicalcium phosphate AR	$CaHPO_4$	29.6	22.4	22.1	0.16	7.31
6	Monocalcium phosphate AR	$Ca(H_2PO_4)_2 \cdot H_2O$	14.2	24.5		24.57	
7	Dicalcium phosphate	$CaHPO_4 \cdot 2H_2O$	23.7	18.0	17.5	0.50	26.25
8	Dicalcium phosphate	$CaHPO_4$	29.9	22.4	21.1	0.24	6.94
9	Dicalcium phosphate USP	$CaHPO_4 \cdot 2H_2O$	28.4	21.9	21.3	0.45	12.3
10	Dicalcium phosphate AR	$CaHPO_4$					7.35
11	Dicalcium phosphate AR	$CaHPO_4$					7.36
12	Dicalcium phosphate AR	$CaHPO_4$					6.94

[1] Loss of weight when sample was ignited in the muffle furnace at 900°C to a constant weight.

material lost only 12.3% of its weight upon ignition as compared with a theoretical loss of 26.17%. The solubility tests of this material (table 1) showed that it contained only a small amount of water-soluble phosphorus, which indicates that the material contained insignificant amounts of monocalcium phosphate. Practically all of the material was soluble in neutral ammonium citrate, which shows the absence of large amounts of tricalcium phosphate. These results indicate that this sample was probably a mixture of $CaHPO_4$ and $CaHPO_4 \cdot 2H_2O$.

Biological studies

Chicks. The purified diet used in these studies was the blood fibrin-gelatin type used routinely in our laboratory (Edwards et al., '58). The unsupplemented diet contained about 0.05 to 0.08% of phosphorus and would not support life in the young chick. Consequently, when phosphorus was added at low levels, the chick's response both for growth and percentage of bone ash was proportional to the amount of available phosphorus added. The various calcium phosphates tested were fed at levels to supply suboptimal amounts of phosphorus in the diet. The Ca:P ratio was maintained at 2:1 in all diets by the addition of $CaCO_3$ when needed. Single Comb White Leghorn cockerels were wing-banded, weighed and housed in electrically heated battery brooders with wire-mesh floors at one day of age. Feed and water were supplied ad libitum. After 21 days, the chicks were weighed and killed and the percentage of bone ash in the tibiae determined as outlined by the AOAC ('55).

Turkeys. The basal diet used in the turkey studies contained the following ingredients expressed as gm/100 gm: dextrose, 25.0; cornstarch, 30.44; blood fibrin, 25.0; gelatin, 5.0; dried buttermilk, 1.0; brewer's dried yeast, 1.0; dehydrated alfalfa meal, 1.0; DL-methionine, 0.1; hydrogenated vegetable fat, 4.5; vitamin A concentrate (5,000 USP units/gm), 0.26; choline chloride, 0.25; NaCl, 0.60; KCl, 0.40; $MgSO_4$, 0.302 plus the following expressed as mg/100 gm; inositol, 22.0; *p*-aminobenzoic acid, 4.4; calcium pantothenate, 2.65; niacin, 6.6; thiamine·HCl, 1.1; riboflavin, 1.1; pyridoxine·HCl, 1.1;

folic acid, 0.22; menadione, 1.1; biotin, 0.44; vitamin B_{12}, 0.005; vitamin D_3 concentrate (15,000 ICU/gm), 8.8; $MnSO_4 \cdot H_2O$, 28.7; $FeSO_4 \cdot 7H_2O$, 57.4; $CuSO_4 \cdot 5H_2O$, 1.8; $CoCl_2 \cdot 6H_2O$, 0.35; $ZnCl_2$, 0.9; KI, 6.2 and $NaMoO_4 \cdot 2H_2O$, 0.11. The diet was analyzed and found to contain 0.09% of phosphorus. The phosphate supplements were incorporated into the diet in place of cornstarch to give the desired phosphorus level and where it was necessary $CaCO_3$ was added to give a Ca:P ratio of 2:1 in all diets. Day-old Beltsville Small White toms were used in the first turkey experiment, and Broad Breasted White toms and hens were used in the second experiment. All the poults were wing-banded and placed in electrically heated battery brooders at one day of age where they had free access to feed and water. After 21 days on experiment, the turkeys were weighed and killed and the tibia removed from the left leg for bone ash determinations (AOAC, '55).

RESULTS AND DISCUSSION

Experiment 1. In this experiment, the availability of the phosphorus to chicks from the calcium phosphate samples, numbers 1 through 6, were determined. The experimental design and the growth and percentage bone ash results of this experiment are presented in table 2. The chicks that received the three tricalcium phosphate samples grew at approximately the same rate. Those that received the NF or AR grade of tricalcium phosphate had higher bone ash values than the chicks receiving comparable levels of β-tricalcium phosphate. Chemical data (table 1) show that the AR appears to be slightly more water-soluble and that both the AR and NF grades contain less citrate-soluble phosphorus.

The USP grade of dicalcium phosphate ($CaHPO_4 \cdot 2H_2O$) appeared to be a very available source of phosphorus to the young chick and was appreciably more available than the phosphorus from AR

TABLE 2

Chick studies on the availability of phosphorus from various types of calcium orthophosphate

	Phosphate description	Phosphorus	Avg wt 3 weeks[1]	Bone ash[1]
		%	*gm*	%
1	β-Tricalcium phosphate	0.20	158	26.4
		0.25	176	31.3
		0.30	191	35.8
		0.35	192	40.2
2	NF tricalcium phosphate	0.20	156	29.9
		0.25	193	34.4
		0.30	193	39.6
		0.35	202	41.4
3	AR tricalcium phosphate	0.20	171	29.8
		0.25	188	32.5
		0.30	178	36.7
		0.35	197	42.8
4	USP dicalcium phosphate	0.20	193	32.7
		0.25	200	37.9
		0.30	209	42.6
		0.35	213	44.6
5	AR dicalcium phosphate	0.20	194	31.5
		0.25	190	35.2
		0.30	196	37.1
		0.35	209	41.7
6	AR monocalcium phosphate	0.20	194	33.1
		0.25	214	38.3
		0.30	201	41.9
		0.35	204	44.6

[1] Single Comb White Leghorn cockerels, 10 chicks/treatment.

dicalcium phosphate ($CaHPO_4$). Here again the analytical data (table 1) show that the dihydrate salt is slightly more soluble in water than the anhydrous material. The phosphorus in monocalcium phosphate, AR grade, was readily available, and was equal to, and perhaps slightly superior to USP dicalcium phosphate as a phosphorus source for chicks.

Experiment 2. In this experiment the availability of the phosphorus in AR tricalcium phosphate, USP and AR dicalcium phosphate and AR monocalcium phosphate to turkey poults was determined. Since we knew from previous experiments in this laboratory that the phosphorus in AR tricalcium phosphate and AR dicalcium phosphate was not readily available to turkey poults, these materials were fed to supply appreciably more phosphorus to the diet than was supplied by either the USP dicalcium phosphate or the AR monocalcium phosphate. The experimental design and results of this experiment are presented in table 3. When 0.55% of phosphorus was added as AR tricalcium phosphate, high mortality and poor bone ash resulted. When the level of supplemental phosphorus was raised to 0.70%, or 0.85%, the percentage mortality was decreased, but the bone ash was not improved significantly. However, the weights and bone ash values from those groups that had high mortality have little meaning since the poults that survive are usually heavier and show higher bone ash values.

The USP dicalcium phosphate was fed to supply lower levels of phosphorus since it was known to be fairly available to young turkeys. However, the 0.25% of phosphorus level was too low and a high mortality ocurred in this group. When 0.40 and 0.55% of phosphorus was supplied as USP dicalcium phosphate mortality was decreased and the rate of growth and percentage bone ash increased appreciably. The AR grade dicalcium phosphate was completely ineffective in preventing mortality when fed at a level to supply 0.85% of phosphorus to the diet. The AR grade monocalcium phosphate proved to be the best source of phosphorus for turkey poults in this study. Not only was mortality reduced, but growth at the lower levels of phosphorus, and bone ash values at all levels of phosphorus were superior to that obtained from the other types of calcium phosphate tested.

Experiment 3. A difference in the availability of the phosphorus from either AR dicalcium phosphate ($CaHPO_4$) or USP dicalcium phosphate ($CaHPO_4 \cdot 2H_2O$) was shown with both chicks and turkey poults. It has been suggested that the difference in availability of these materials might be explained on the purity of the products instead of the difference in physical structure and chemical properties. Therefore, the two products, samples 7 and 8, were

TABLE 3

Results of the turkey experiment comparing various types of calcium orthophosphate

	Phosphate description	Phosphorus	Avg wt 3 weeks[1]	Bone ash[1]	Mortality
		%	*gm*	%	%
3	AR tricalcium phosphate	0.55	242	30.5	80
		0.70	246	34.6	30
		0.85	236	33.5	10
4	USP dicalcium phosphate	0.25	207	25.4	80
		0.40	234	34.9	20
		0.55	316	39.0	0
5	AR dicalcium phosphate	0.55	—	—	100
		0.70	121	20.9	90
		0.85	—	—	100
6	AR monocalcium phosphate	0.25	217	31.6	30
		0.40	298	42.4	0
		0.55	305	48.7	0

[1] Small White Beltsville toms, 10 poults/treatment.

TABLE 4

Effect of specially prepared dicalcium phosphates on the growth and bone ash of chicks and poults

Phosphate sample		Supplemental phosphorus	3-Week wt	Bone ash	Mortality
		%	*gm*	%	%
No. chicks[1]					
7	$CaHPO_4 \cdot 2H_2O$	0.20	206	36.2	0
		0.30	213	42.7	0
8	$CaHPO_4$	0.20	182	33.7	0
		0.30	214	41.9	10
No. turkeys[2]					
7	$CaHPO_4 \cdot 2H_2O$	0.40	267	38.4	0
		0.55	247	43.4	0
8	$CaHPO_4$	0.70	256	35.3	14
		0.85	277	42.2	14

[1] Single Comb White Leghorn cockerels, 10 chicks/treatment.
[2] Broad Breasted White poults, 7 toms and 7 hens/treatment.

prepared in the laboratory from reagent grade chemicals, using distilled water. The solubility fractionation and loss of weight on ignition (table 1) indicated that they were pure preparations of $CaHPO_4 \cdot 2H_2O$ and $CaHPO_4$. These samples were tested in an experiment and the results are presented in table 4. The results of the chick study indicate that the phosphorus in the dihydrate form ($CaHPO_4 \cdot 2H_2O$) is slightly more available than that in the anhydrous form ($CaHPO_4$). The results of the turkey experiment show a marked difference in availability of the two sources of phosphate. However, mortality in the groups receiving the $CaHPO_4$ was not as severe as in previous studies (table 3) and may be due to the different breed of poults used in this experiment. The percentage bone ash values are lower for the poults receiving the $CaHPO_4$ even though the phosphorus was supplied at higher levels. These results demonstrate the poor availability of the phosphorus in this type of calcium phosphate for turkey poults.

The results obtained in this experiment show that the difference in the availability of the phosphorus in dihydrate and anhydrous dicalcium phosphate is probably due to differences in physical structure or chemical properties of the materials and not to impurities in the USP samples. The differences in availability of the phosphorus from various types of phosphorus do not appear to be correlated with the solubility tests conducted except that the more water-soluble compounds tended to be more available. These results demonstrate the marked difference between chicks and poults in their ability to utilize phosphorus from various forms of calcium phosphate. The reason for this difference is unknown; however, physiological differences between the chick and the turkey, such as the differences in pH of the gastrointestinal tract, rate of absorption or length of time that food is held in various parts of the gastrointestinal tract might account for the observed differences in the ability of the two species to utilize these phosphate materials.

SUMMARY

Experiments were conducted to study the availability of phosphorus in different chemical grades and various types of calcium orthophosphate to chicks and turkey poults.

In general the primary calcium phosphate salt is the most available, followed by the secondary, with the tertiary salt being the least available. However, the degree of hydration and resulting physical and chemical form of the secondary salt also affects the degree of availability, with the dihydrated salt being more available than the anhydrous form.

It was shown that the chick does not differentiate to the same degree as the turkey poult between the various chemical

grades and forms of calcium phosphate. In particular, the turkey poult utilized the anhydrous form of dicalcium phosphate much less efficiently than the dihydrate form. Thus anhydrous dicalcium phosphate is not the preferred source of dietary phosphorus for this species.

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Hematological Studies in Mature Turkey Hens Fed Diets Deficient in B Vitamins[1,2]

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Boucher et al. ('41–'42) reported riboflavin to be essential for hatchability of turkey eggs. Pantothenic acid and folic acid requirements for hatchability in turkeys were studied by Kratzer et al. ('55, '56). These are the only B vitamins for which the National Research Council ('60) lists quantitative requirements for adult turkeys. Since much more work has been reported on the requirements of chickens, many estimates of requirements are based on the assumption that the response of the turkey is similar to that of the chicken.

Recent work (Ferguson et al., '61) showed that reproductive performance and hematological changes may be affected by deficiencies of several B vitamins including riboflavin, pyridoxine, and pantothenic acid. The following experiment was designed to study further the dietary need for riboflavin, pyridoxine, and pantothenic acid and the effect of these vitamins on blood characteristics in the mature turkey hen.

MATERIALS AND METHODS

Forty-eight Beltsville Small White turkey hens, which were reared on range, were randomly divided into 8 groups of 6 birds each and placed in individual laying cages. The synthetic control diet which was fed to 12 birds was the same as that used by Ferguson et al. ('61). Groups of 6 birds each were fed the synthetic diet deficient in riboflavin, pyridoxine, or pantothenic acid or containing very low-level supplements of the vitamins as indicated in table 1. In the diets termed "deficient," all of the vitamin in question was omitted from the basal diet. The diets termed "low" had suboptimal levels of the vitamin added; e.g. the low-riboflavin diet contained 0.3 mg/454 gm, the low-pyridoxine diet contained 0.3 mg/454 gm, and the low-pantothenic acid diet contained 1.5 mg of calcium pantothenate/454 gm. Since quantitative requirements of adult turkeys for pyridoxine are unknown, the level in the pyridoxine diet was approximately 25% of the estimated requirement based on the quantities of pyridoxine commonly added to turkey diets. The "low" levels of riboflavin and pantothenic acid in the diet were 20% of the minimum requirement according to the National Research Council ('60).

After the experimental diets were fed for 12 weeks, three hens of the riboflavin-deficient group, two hens of the low-riboflavin group and two hens of the low-pyridoxine group were fed the basal diet that contained all vitamins, to permit recovery from the deficiencies in an attempt to affect resumption of egg production (table 1). Feed and water were supplied ad libitum.

Yolks from eggs laid at the beginning and near the end of the experiment were assayed microbiologically for riboflavin, pyridoxine and pantothenic acid. Riboflavin was assayed by the method of Snell and Strong, ('39), pantothenic acid was assayed by the method of Skeggs and

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TABLE 1

Influence of deficiencies of riboflavin, pyridoxine and pantothenic acid on hematological characteristics of adult turkey hens

Group	Diet	Hemoglobin	Hematocrit	Erythrocyte[1]	MCV[2]	MCH[3]	MCHC[4]
		%	%	*millions/mm³*	*mm³/10⁷ cells*	*mg/10⁷ cells*	*gm/100 ml*
1	Control	11.38(12)	39.3(12)	2.62(12)	148(12)	43.4(12)	28.9(12)
2	Riboflavin-deficient, 12 weeks; control 5 weeks	9.53(3)	34.7(3)	1.90(3)	183(3)	50.2(3)	29.3(3)
3	Riboflavin-deficient	8.52(3)	30.3(3)	2.00(3)	150(3)	42.6(3)	29.1(3)
4	Low riboflavin, 12 weeks; control 5 weeks	10.61(2)	37.5(2)	2.11(2)	178(2)	50.3(2)	31.2(2)
5	Low riboflavin	11.90(3)	40.7(3)	2.23(3)	183(3)	53.4(3)	29.2(3)
6	Pyridoxine-deficient	11.08(3)	38.7(3)	2.28(3)	170(3)	48.6(3)	28.7(3)
7	Low pyridoxine	11.04(3)	40.7(3)	2.35(3)	173(3)	47.0(3)	27.2(2)
8	Low pyridoxine, 12 weeks; control 5 weeks	10.12(2)	37.0(2)	2.27(2)	168(2)	44.6(2)	27.4(2)
9	Pantothenic acid-deficient	11.89(6)	40.0(6)	2.27(6)	176(6)	52.4(6)	29.7(6)
10	Low pantothenic acid	10.39(5)	35.6(5)	2.24(5)	159(5)	46.3(5)	29.2(5)

[1] Treatment main effect was significant at the 0.01 level of probability. Numbers in parentheses indicate the number of determinations/group.
[2] MCV indicates mean cell volume.
[3] MCH indicates mean cell hemoglobin.
[4] MCHC indicates mean cell hemoglobin concentration.

Wright ('44), and pyridoxine by the method of Barton-Wright ('45).

In the sixteenth week of the experiment, blood samples were drawn from the wing veins of the birds for the determination of hemoglobin, packed cell volume (hematocrit), erythrocyte count, and electrophoretic migration of plasma proteins. Mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) also were determined. Electrophoretic migration of plasma proteins was determined in a Spinco model R paper strip electrophoresis apparatus using veronal buffer of pH 8.6. Heparin was used as an anti-coagulant. Total plasma protein was determined spectrophotometrically by the method of Eichel and Swanson ('57). Data were analyzed statistically by the analysis of variance method according to Snedecor ('56). Because of unequal numbers of birds in the groups, the means were not separated.

RESULTS AND DISCUSSION

The erythrocyte counts were less in all deficient and low-vitamin supplemental groups than in the control group (table 1). Counts for the deficient and low groups ranged from 1.90 to 2.35 million/mm³, compared with 2.62 million/mm³ in the control group. Riboflavin-deficient and low groups, the pyridoxine-low group and the pyridoxine-low group that received the control diet for the last 5 weeks showed no improvement in erythrocyte count when compared with the corresponding groups that continued to be fed the deficient and low-vitamin supplemented diets. These results agree closely with the results of Ferguson et al. ('61). Erythrocyte counts were significantly different at the 0.01 level of probability.

Hemoglobin values were lower in the riboflavin-deficient groups (2 and 3) than in the control group (table 1). Group 2, which received the control diet for the last 5 weeks of the experiment, had 9.53% hemoglobin compared with 8.52% for the group 3 that was fed the riboflavin-deficient diet for the entire experiment. This indicates partial recovery of group 2 from the deficiency. Hemoglobin for groups 4 to 10 were essentially the same as that of

the control group. The differences observed in hemoglobin values were not statistically significant.

The hematocrit of the group receiving the riboflavin-deficient diet was 30.3%, compared with 39.3% for the group receiving the control diet (table 1). Partial recovery from riboflavin deficiency symptoms is indicated again in group 2. Slight variations in hematocrit were noted in other groups but differences were not significant.

The mean cell volumes were greater in all deficient and trace groups, when compared with the control group, with the exception of group 3 which received the riboflavin deficient diet for the entire 17 weeks (table 1). The value for group 2 was comparable to the values for the less severe riboflavin trace diets. The effect of dietary treatments on MCV closely approached statistical significance at the 0.05 level of probability (F values of 2.10 vs. 2.19).

The mean cell hemaglobin values (MCH) were greater in all groups receiving deficient and trace diets than in the control group, with the exception of group 2, in which the values were approximately equal to those of the control group (table 1). Groups 4 and 8 fed the basal diet during the last 5 weeks, showed some reduction in MCH when compared with corresponding groups (5 and 7) which had been fed diets deficient in riboflavin and pyridoxine, respectively, throughout the 17-week period. Differences in MCH were not found to be statistically significant. Mean cell hemoglobin concentration (MCHC, table 1) and total plasma protein determinations (table 2) showed some variations but differences were not statistically significant.

The ratio of albumin to globulin (A/G) in group 3, (riboflavin-deficient) was 0.31 (table 2). Riboflavin-deficient hens fed the basal diet for 5 weeks (group 2) had an A/G ratio of 0.41; the A/G ratio in the control group was 0.53. Feeding the basal diet to group 2 resulted in a slight increase in albumin and a decrease in globulin, when compared with the riboflavin-deficient group. The globulin fraction was lower than that of the control hens in groups 4, 9 and 10 (low-riboflavin followed by control, pantothenic acid-deficient and low-pantothenic acid diets, respectively). Albumin was decreased in group 7 fed the

TABLE 2

Influence of deficiencies of riboflavin, pyridoxine and pantothenic acid on albumin and globulin levels in plasma of mature turkey hens

Group	Diet	Total protein	Albumin	Globulin	A/G[1] ratio
		mg/ml	*mg/ml*	*mg/ml*	
1	Control	52.8(12)	17.9(12)	34.9(12)	0.53(12)
2	Riboflavin-deficient 12 weeks; control 5 weeks	50.5(3)	14.7(3)	35.8(3)	0.41(3)
3	Riboflavin-deficient	50.6(3)	12.0(3)	38.6(3)	0.31(3)
4	Low riboflavin, 12 weeks; control 5 weeks	45.7(2)	18.0(2)	27.7(2)	0.65(2)
5	Low riboflavin	51.9(3)	20.1(3)	31.8(3)	0.63(3)
6	Pyridoxine-deficient	53.9(3)	18.7(3)	35.2(3)	0.53(3)
7	Low pyridoxine	52.2(3)	15.6(3)	36.6(3)	0.44(3)
8	Low pyridoxine, 12 weeks; control 5 weeks	51.5(2)	17.4(2)	34.1(2)	0.51(2)
9	Pantothenic acid-deficient	47.6(6)	19.8(6)	27.8(6)	0.71(6)
10	Low pantothenic acid	47.3(5)	20.6(5)	26.7(5)	0.84(5)

[1] Treatment main effect was significantly different at the 0.01 level of probability. Numbers in parentheses indicate the number of determinations/group.

pyridoxine trace diet, which resulted in an A/G ratio of 0.44.

The A/G ratio was higher than that of the control groups in hens fed the low-riboflavin diets (groups 4 and 5), pantothenic acid deficient and low-pantothenic acid diets (groups 9 and 10). No difference in the A/G ratio was found between the pyridoxine deficient group and the control group or the group fed the pyridoxine-deficient diet followed by the control diet (group 8).

Egg production decreased greatly in all groups receiving deficient and trace diets except in group 10 which was fed the diet containing 1.5 mg of pantothenic acid/454 gm of diet (table 3). Thus it appears that 1.5 mg of pantothenic acid/454 gm of feed is sufficient for maximal egg production under the conditions of this experiment. Egg production in the entire experiment was far below that which would be expected from birds fed a practical-type diet, as was the case in the practical control group of Ferguson et al. ('61).

In the third week of egg production the riboflavin level of egg yolks from hens receiving the riboflavin trace diet had declined to zero, compared with 0.68 mg/gm for the control group. By the third week egg production had ceased in the riboflavin-deficient group. By the eighth week egg production had ceased in the low-riboflavin group.

Egg yolks from the groups receiving pyridoxine-deficient and low-pyridoxine diets contained 1.32 and 2.02 mg of pyridoxine/gm, respectively, compared with 2.89 mg/gm in yolks from the control group during the third week of egg production. After 8 weeks, pyridoxine content of egg yolks from the deficient and low-pyridoxine groups had decreased in pyridoxine content by approximately 50%, whereas that of eggs from the control group was only slightly below the value obtained at the start of the experiment. The control group had a value of 2.62 mg/gm compared with 0.75 and 0.88 mg/gm in the deficient and low-pyridoxine groups, respectively.

The pantothenic acid content of egg yolks from the group receiving the control diet was essentially unchanged during the 8 weeks of the production period (112 mg/gm of yolk in the third week; 108 mg/gm in the eighth week). The group receiving the pantothenic acid-deficient diet showed a decline in yolk content from 21 mg/gm to 15 mg/gm for the same period of time. The group receiving the low-pantothenic acid diet had corresponding values of 42 and 39 mg/gm of yolk.

Embryonic mortality in the first week of incubation was very high in eggs from birds fed the deficient diets (groups 2, 6, and 9) as well as in eggs from those fed the riboflavin trace diet (table 3). Most of the live embryos were removed at 7.5 days of incubation for tissue enzyme determinations, so that valid hatchability data could not be obtained. Results from the few embryos that were permitted to remain for the complete incubation period were not in variance with the requirements for riboflavin, pyridoxine, and pantothenic acid for hatchability of turkey eggs as reported by Ferguson et al. ('61).

TABLE 3

Effect of deficiencies of riboflavin, pyridoxine and pantothenic acid on egg production, embryonic mortality, and feed consumption by mature turkey hens

Group	Diet	Weeks of production				Embryonic mortality	Feed consumed/hen/day
		0–4	5–8	9–12	13–16[1]	1–7 days	
		eggs/hen	*eggs/hen*	*eggs/hen*	*eggs/hen*	*%*	*gm*
1	Control	4.92	5.75	4.25	1.25	12	104.4
2 and 3	Riboflavin-deficient	0.67	0	0	0	100	86.3
4 and 5	Low riboflavin	1.83	1.33	1.00	0	94	95.3
6	Pyridoxine-deficient	1.50	1.67	1.17	0.83	59	81.7
7 and 8	Low pyridoxine	7.67	1.17	0.17	0.50	4	99.9
9	Pantothenic acid-deficient	2.50	1.83	2.00	0	58	109.0
10	Low pantothenic acid	5.17	5.17	4.33	1.33	18	118.0

[1] No eggs were produced in the 17th week.

Appetite was depressed in the riboflavin and pyridoxine-deficient groups compared with that of the control group (table 3). Feed consumption in the diets supplemented with low levels of the vitamins being investigated was greater in every case than in the corresponding deficient diets. The hens fed the low-pantothenic acid diet ate more feed per hen per day than did the hens in the control group. These results agree with the report of Ferguson et al. ('61), in that riboflavin and pyridoxine deficiencies result in a decrease in feed consumption.

SUMMARY

Beltsville Small White turkey hens maintained in individual cages were fed diets deficient in riboflavin, pyridoxine, or pantothenic acid; other groups were fed diets containing suboptimal levels of these vitamins.

Erythrocyte counts of the hens from the groups fed the deficient diets or diets with low levels of the vitamins, were from 10.3 to 27.5% below that of the group that received the complete synthetic diet. Hemoglobin and hematocrit values were lower in the riboflavin-deficient group than in the control group. However, differences observed in hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin concentration and plasma proteins were not statistically significant. Albumin:globulin ratios were slightly higher in groups receiving diets low in riboflavin and pantothenic acid or deficient in pantothenic acid than in the control group. The albumin:globulin ratio of the group receiving the riboflavin-deficient diet was lower than that of the control group.

Hens fed the deficient diets or diets containing low levels of riboflavin, pyridoxine or pantothenic acid produced eggs with lower levels of the respective vitamin in the yolk than the control group. Egg yolks from the hens receiving low levels of the vitamins contained greater amounts than did the egg yolks from hens receiving the deficient diets. Egg production was increased in groups fed low levels of the vitamins when compared with egg production in groups fed deficient diets. Riboflavin and pyridoxine deficiencies appeared to decrease the appetite of the hens.

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Effect of Diets with and Without Fat at Low and High Caloric Levels on Fatty Acids in Blood Cells and Plasma of Dogs[1]

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When diets deficient in linoleic acid are fed, low concentrations of dienoic and tetraenoic acids and a high concentration of trienoic acid have been found in the blood serum or plasma in young infants (Hansen et al., '58); in puppies (Wiese and Hansen, '51); in rats (Mead, '57; Holman, '60); and in monkeys (Greenberg and Moon, '61). In addition a marked increase in trienoic acid with moderate decreases in di- and tetraenoic acids in red blood cells in fat deficiency have been observed in rats by Holman ('60) and in monkeys by Portman et al. ('61) Greenberg and Moon ('61) and Fitch et al. ('61). Wiese et al. ('62) have reported that manifestations of fat deficiency in puppies were aggravated and lessened, respectively, by high and low caloric intakes, which features were correspondingly reflected in the changing values for di- tri- and tetraenoic acid levels in the blood serum. It seemed pertinent, therefore, to determine the distribution of saturated and unsaturated fatty acids in red blood cells and in plasma of animals receiving diets with and without fat when fed at different caloric intakes.

MATERIALS AND METHODS

Experimental conditions. Beagle puppies (7 to 11 weeks of age) were given a diet either very low in fat (< 1% of the calories) or one in which fat comprised 15% of the caloric intake, the fat being substituted isocalorically for sucrose. Protein derived from skim milk powder and casein provided 15% of the calories, although in three of the 24 animals 20% of the calories were given as protein. The source of fat was fresh steam-distilled lard which on analysis by gas-liquid chromatography had the following composition expressed as per cent of the total fatty acids: lauric, 0.1; myristic, 1.2; palmitic, 24.4; palmitoleic, 3.4; stearic, 14.9; oleic, 44.8; linoleic, 9.9; and linolenic, 1.3. Supplements of minerals, cellulose and a multivitamin preparation were given in the manner described by Wiese et al. ('62). Twelve puppies were fed the low-fat diet and another 12 receiving fat, served as controls. From weaning up to 4 months of age, littermates were given weighed amounts of food that were considered to be at low, medium and high caloric levels, namely, 100, 150 and 200 Cal./kg/day, respectively. After 4 months of age, the intakes were lowered by 15%. The animals were the same as those used by Wiese et al. ('62); however, the time intervals for obtaining blood specimens were different.

Methods of procedure. Fasting blood samples were obtained at two to seven months after the animals had been supplied with the experimental diets, which was at the time of weaning. Heparin was used as the anticoagulant. The specimens were centrifuged at 2,000 rev/min for 15 minutes and the plasma withdrawn for analysis. The cells were washed three times with an isotonic saline solution and centrifuged at 2,000 rev/min after each washing. The buffy layer was removed by capillary pipette. Then 3 ml of red blood cells were measured and an equal volume

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of distilled water was added so as to lake the cells. Fat was extracted from the laked cells using a hot mixture of alcohol-ether (3:1) and the filtered extracts saponified with alcoholic KOH. After removal of the unsaponifiable fraction, the total fatty acids were determined semi-microgravimetrically by the technique of Wilson and Hansen ('36). The same procedure was used for the preparation of total fatty acids from the plasma. The di- tri- and tetraenoic acids were determined by alkaline isomerization, and in addition, aliquots containing approximately 1 to 2 mg of fatty acids were methylated at 70°C with 2% sulfuric acid in absolute methanol. Methyl esters were chromatographed in a Beckman GC-2 instrument at 220°C on a 12-foot (3.66-meter) diethylene glycol succinate column with helium as the carrier gas. The quantities of saturated and unsaturated fatty acids were calculated by comparison with areas obtained from known amounts of specific fatty acids.

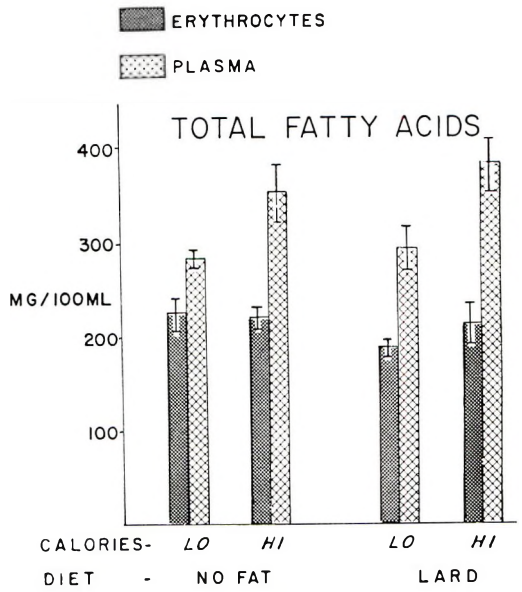

Fig. 1 Total fatty acids with standard deviations in the erythrocytes and plasma in puppies fed at low (Lo) and high (Hi) caloric intakes for 6 to 7 months after weaning.

RESULTS

Inasmuch as the findings for di- tri- and tetraenoic acids by alkaline isomerization and by gas chromatography compared very well, only the results by the latter method are being presented. Analyses of fatty acids in the erythrocytes and plasma were conducted on the dogs fed at three caloric levels; however, the distribution of saturated and unsaturated fatty acids in blood cells and plasma for the dogs fed at the medium (normal) caloric intake was quite similar to that of those fed at the high caloric level after receiving the same diets for 6 to 7 months. Hence, in figures 1, 2 and 3 the results with standard deviations are given only for the dogs that received low and high caloric intakes with and without fat in the diet.

Total fatty acids. In figure 1 are presented summary data concerning the mean values with standard deviations for total fatty acids in the red blood cells and in the plasma from the animals after having been fed diets with and without fat at two caloric levels for a period of 6 to 7 months after weaning. The level of total fatty acids in plasma was proportional to the caloric intake and was greater than in the cells with or without fat (lard) in the diet. In the erythrocytes, the level of the total fatty acids did not appear to be influenced by the caloric intake.

Di- tri- and tetraenoic acids. As indicated in figure 2, with the diet deficient in fat, the dienoic (linoleic) acid level was markedly less, both in cells and plasma, than when lard was present in the diet. Also the content of dienoic fatty acid was much less in the erythrocytes than in the plasma. This was true for both groups of animals (with and without fat). Dogs with low caloric intakes had slightly more linoleic acid in their red cells and plasma than did the littermates that consumed twice as many calories.

Trienoic acid, the presence of which is so characteristic of the fat-deficient state, was high in both the cells and plasma of all animals fed the low-fat diet. Although there was little difference between the levels in red cells and plasma, the effect of caloric intake was very marked. With the greater rate of growth resulting from greater food consumption, there was a larger accumulation of trienoic acid both in the cells and in plasma. No trienoic acid was found in the red blood cells when fat was present in the diet.

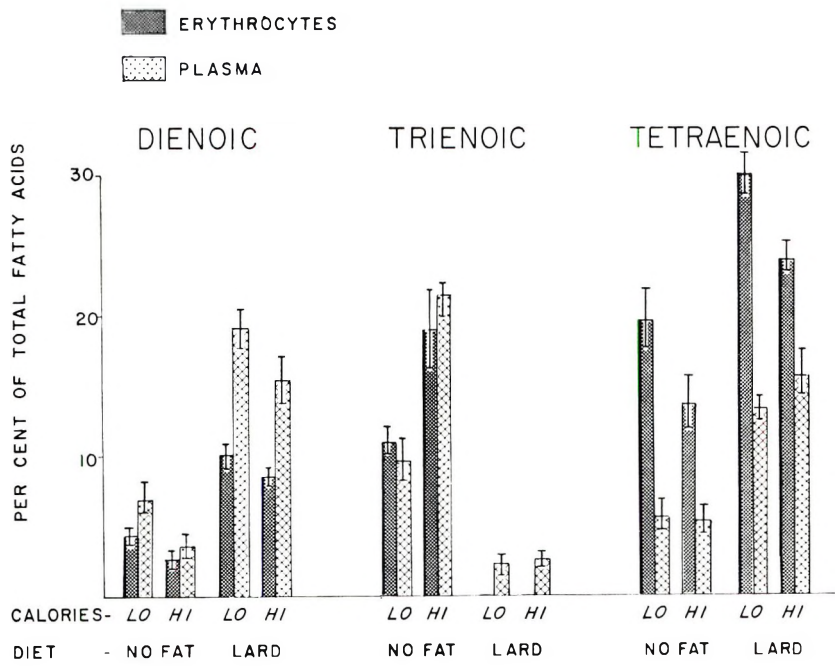

Fig. 2 Di- tri- and tetraenoic acids with standard deviations in the erythrocytes and plasma of fat deficient and control puppies fed at low (Lo) and high (Hi) caloric intakes for 6 to 7 months after weaning.

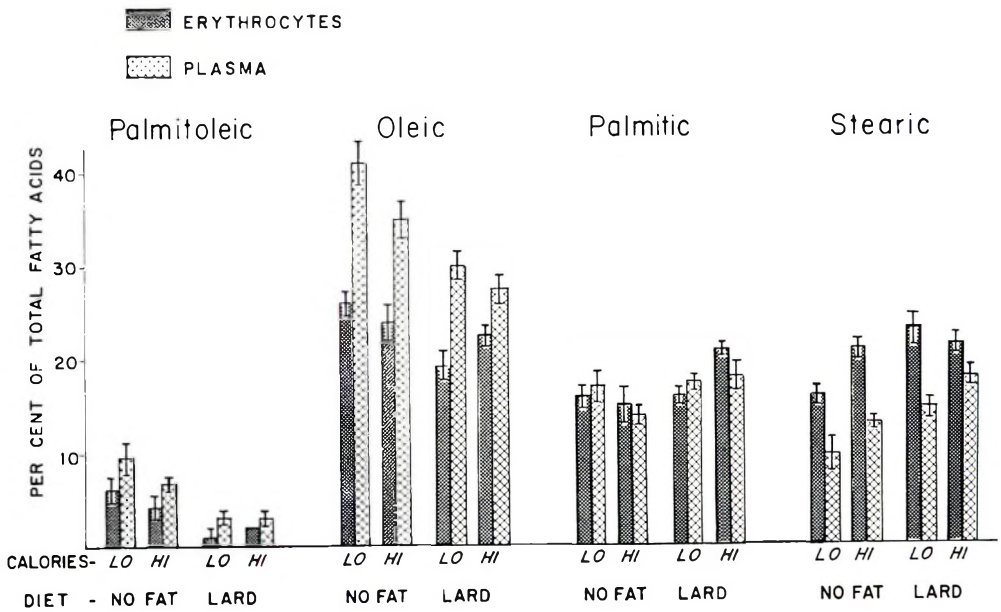

Fig. 3 Monoene and saturated fatty acids with standard deviations in the erythrocytes and plasma of puppies fed at low (Lo) and high (Hi) caloric intakes for 6 to 7 months after weaning.

A reverse effect of caloric intake from that observed for trienoic acid was evident for tetraenoic (arachidonic) acid in the red cells. Higher levels for tetraenoic acid were observed when the intake of calories was low. Such was found to be the case in both fat-deficient and control animals. Plasma levels for tetraenoic acid were affected by caloric intake to a much smaller degree than were the red cells. Characteristically tetraenoic acid levels were lowest in the fat-deficient state.

Monoene and saturated fatty acids. Without fat in the diet, as indicated in figure 3, the levels of the monoene fatty acids (palmitoleic and oleic) were higher in both plasma and cells than when fat was present in the diet. The levels were somewhat higher in plasma than in cells, regardless of caloric intake.

The trends for the levels of the saturated fatty acids, palmitic and stearic, were not consistent with respect to caloric intake or absence of dietary fat. Stearic acid was always higher in cells than in plasma.

Changes in relation to time. Even though there were no visible alterations in the appearance of the skin of the puppies fed the low-fat diet for two months, most of the changes in distribution of the fatty acids which were evident in the erythrocytes and plasma after 6 to 7 months also were detectable at two months after weaning. The magnitude of these early changes in the di- tri- and tetraenoic acids is depicted at three caloric levels in figure 4. The dienoic acid levels were low both in cells and plasma but by two to three months had not reached the minimal level observed after the puppies had been fed the fat-deficient diet for 6 to 7 months. At two months the trienoic acid definitely was increasing in the cells and plasma but the increases were much greater in plasma than in cells. The amount of trienoic acid in both compartments was directly proportional to the number of calories consumed. Marked decreases in the percentages of tetraenoic acid were noted at all caloric levels in plasma. There was no reduction in the tetraenoic acid level in erythrocytes of puppies fed at the low caloric intake. There were definite decreases, however, at the high caloric intakes. The monoene fatty acids, palmito-

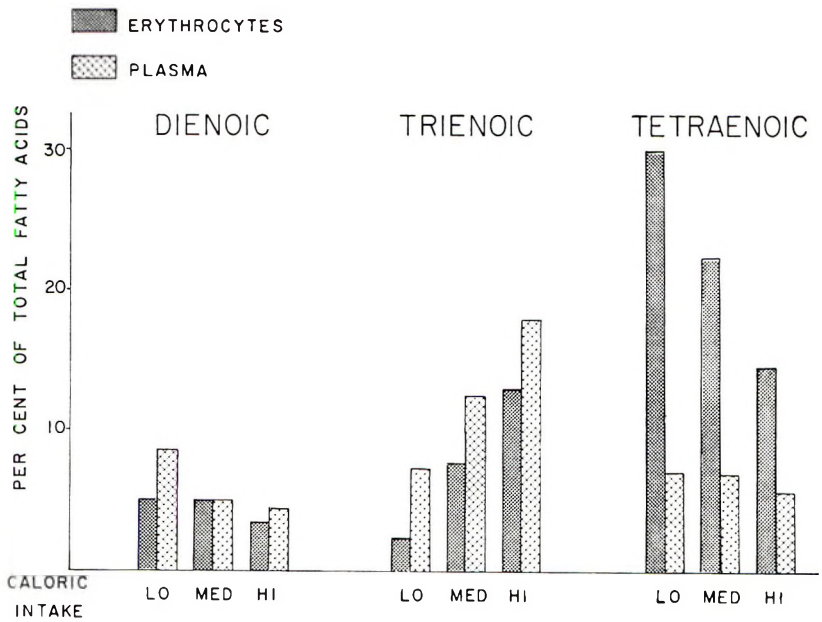

Fig. 4 Di- tri- and tetraenoic acids in erythrocytes and plasma of puppies after being fed low-fat diet at low (Lo) medium (Med) and high (Hi) caloric intakes for two to three months.

leic and oleic, also showed increases in amount in cells and plasma after two months of feeding of the low-fat diet.

DISCUSSION

For the young growing dog, diets low in linoleic acid were associated uniformly with low values for dienoic and tetraenoic acids and high levels for a trienoic acid both in the red blood cells and in plasma. These observations confirm those of other workers for the rat and the monkey.

Greenberg and Moon ('61) did not observe trienoic acid in blood cells of monkeys after feeding a low-fat diet for two months, however, in our study with young puppies this fatty acid appeared in the erythrocytes as early as two months after the low-fat diet was fed. Dietary effects on the lipids of blood cells and plasma were most pronounced in the rapidly growing animals that were fed at a high caloric level. Also the early signs of fat deficiency were more pronounced for the tri- and tetraenoic acids in plasma than in the cells. Although it is possible that puppies are more susceptible to a lack of dietary fat containing linoleic acid than are monkeys, it appears more likely that the rate of appearance of trienoic acid in red cells as reported by Greenberg and Moon ('61) differed from that observed in our puppies due to differences in the age of the animals under study. Rate of growth would be influenced by the age of the animals. Wiese et al. ('62) demonstrated the marked effect of caloric intake and rate of growth on the rate of development of fat deficiency signs and symptoms. Also, in very young infants who were fed a milk mixture low in linoleic acid Wiese et al. ('58) noted definite decreases in the di- and tetraenoic acids and an increase in the trienoic acid in serum after one to two weeks. It appears from the rapid changes which take place in serum and plasma levels for the di- tri- and tetraenoic acids that an earlier evaluation of the nutritional state of an animal with respect to adequacy of fat intake can be obtained from a study of plasma or serum than from the cellular portion of the blood. However, this does not minimize the importance of the cellular changes that take place.

SUMMARY

In a study with 24 Beagle puppies receiving diets with and without fat (lard) for 6 to 7 months after weaning, it was found that the distribution of the fatty acids in erythrocytes and in plasma was influenced by the level of the caloric intake in the following respects.

(1) The dienoic and tetraenoic acids were low in the red blood cells and plasma in the animals fed the low-fat diets as compared with the control animals that were fed fat. At the high caloric intakes the levels for both di- and tetraenoic acids were always less (cells and plasma) with and without fat than at the low caloric intakes. Dienoic acid levels were always lower in cells than in plasma whereas tetraenoic acid levels were consistently greater in cells than in plasma.

(2) Trienoic acid levels were higher in blood cells and plasma in the absence of dietary fat. The level of this fatty acid in the fat-deficient state was proportional to the caloric intake. When fat was lacking in the diets there was little difference in levels between the cells and plasma, whereas in the control animals receiving fat no trienoic acid was found in the red cells.

(3) Palmitoleic and oleic acids were higher in plasma and blood cells of the animals fed the low-fat diet than in the control group. These levels were high in plasma compared with blood cells, irrespective of caloric intake.

(4) The saturated fatty acids (palmitic and stearic) were not altered significantly by caloric intake or level of fat in the diet. Stearic acid was higher in blood cells than in plasma.

(5) In both red cells and plasma definite changes in the amounts of the di- tri- and tetraenoic acids in fat-deficient animals were evident as early as two to three months of feeding time. Dienoic acids decreased greatly but the drop was more marked at the high caloric level. Increases in trienoic acids were directly proportional to the caloric intake, whereas the levels for tetraenoic acids were inversely proportional to the caloric intake. The magnitude of changes in tri- and tetraenoic acids at two to three months was greater in plasma than in cells.

It was concluded from study of the serum lipids in the fat-deficient state that the dienoic acid levels of both cells and plasma are reliable indices of the dietary lack of linoleic acid. Moreover, the synthesis of trienoic acid and of tetraenoic acid are influenced remarkedly by the caloric intake and the resulting rate of growth; hence, they are also of value in interpreting degrees of deficiency.

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Effect of Certain Factors on Nitrogen Retention and Lysine Requirements of Adult Human Subjects

II. INTERVAL WITHIN EXPERIMENT WHEN DIETARY LYSINE AND NITROGEN WERE CONSTANT[1]

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Determination of amino acid requirements of human subjects necessitates the administration within a single experiment of different quantities of the amino acid under investigation in order to ascertain the smallest quantity that will maintain nitrogen equilibrium (Rose, '57; Leverton, '60; Clark et al., '60). With such an experimental design, however, the effectiveness of the minimal amount of a particular amino acid usually can be tested for only a few days. Estimates of amino acid requirements established in short periods should be supported by data obtained when quantities of the amino acid approximating minimal needs are consumed for several consecutive weeks, together with adequate amounts of other essential amino acids and of nitrogen.

Three experiments therefore were conducted to examine the applicability of lysine requirements previously estimated in short-term experiments in this laboratory (Clark et al., '60), and to observe directional trends that might develop when dietary lysine was near but not necessarily equal to minimal needs of individual subjects. The lysine intake of all subjects was held constant throughout each experiment at one of three selected quantities. Total amounts of other essential amino acids and of nitrogen were the same as those provided when minimum lysine requirements were established.

PROCEDURE

The procedures for investigation of nitrogen metabolism were similar to those described in earlier publications (Clark et al., '57, '60) except as indicated.

Subjects. The graduate students or upper classmen who participated were between 20 and 30 years of age and were judged healthy by a physician. The women weighed between 56.1 and 67.4 kg, and the men between 62.0 and 75.9 kg (table 1). Subjects EO, ER and JY served in two experiments.

Dietary nitrogen and calories. The total daily nitrogen intake of each subject was approximately 9.0 gm, of which 45% was supplied by 159 gm of white wheat flour, 21 gm of cornmeal and a few low-nitrogen foods; 9% by a mixture of essential amino acids; and 46% by glycine, glutamic acid and diammonium citrate, each of which contributed one-third of the nitrogen. The quantities of essential amino acids, cystine and tyrosine provided by the basal diet and the amino acid mixture were equivalent to those present in 20 gm of egg protein. The diet was supplemented with vitamins[3] and a mineral mixture.

Information concerning the basal caloric expenditure and physical activity of each subject was used in making preliminary estimates of caloric requirements which were modified as necessary during the

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[1] Journal paper 1914, Purdue Agricultural Experiment Station, Lafayette, Indiana. This contributes to the North Central Regional Cooperative Project NC-49, factors affecting requirements of adult human subjects for protein and amino acids. A preliminary report was presented before the American Institute of Nutrition in 1959.

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[3] The authors gratefully acknowledge the Litrison vitamin capsules contributed by Hoffmann-La Roche, Inc.

TABLE 1

Description of subjects

Lysine intake	Subject	Sex	Age	Body weight	Height	Surface area	Caloric intake[1]	Creatinine
mg			*years*	*kg*	*cm*	m^2		*gm*
600	ER	F	22	65.0	175	1.78	2700	1.33
	CS	M	21	64.9	172	1.73	3200	1.54
	JY	M	20	62.8	185	1.80	3350	1.45
	MZ	M	21	74.4	177	1.88	3250	1.67
700	PG	F	22	65.9	165	1.71	2600	1.31
	WM	M	24	62.0	181	1.81	3550	1.61
	EO	M	26	65.0	183	1.84	3600	1.82
	ER	F	22	67.4	174	1.78	2700	1.51
	BS	F	25	56.1	168	1.61	2450	1.07
	FS	F	22	56.3	167	1.61	2450	1.19
950	WB	M	23	75.9	187	1.98	3500	1.55
	GD	M	21	64.4	178	1.85	3100	1.41
	EO	M	26	65.9	183	1.84	3600	1.61
	RP	M	25	70.6	178	1.85	3500	1.83
	JY	M	20	64.4	185	1.80	3300	1.49

[1] Includes 150 Cal. estimated to be supplied by purified amino acids.

12-day adjustment period at the beginning of the experiment. The total caloric intakes shown in table 1, which include an estimated 150 Cal. from the amino acid mixtures, maintained initial body weight and were continued throughout the experiment except for adjustments of 5% or less for three subjects. The women consumed between 2,450 and 2,700 Cal., and the men between 3,100 and 3,600 Cal.

Lysine intakes. Since time did not permit determination of minimum lysine requirements of individual participants, data obtained in earlier experiments were utilized. The mean lysine requirement for maintenance of nitrogen equilibrium of 9 men who weighed between 62.6 and 86.2 kg was 700 mg with a range of 400 to 900 mg, and the mean requirement of 10 women weighing from 49.9 to 79.8 kg was 550 mg with a range of 300 to 700 mg (Clark et al., '60). None of the minimum lysine requirements of participants in the current series, as predicted from body weight, surface area or creatinine excretion (table 1), exceeded the upper limits established previously.

The total amounts of lysine consumed in these three experiments were believed to be equivalent to the minimum requirements of some subjects and above or below the needs of others. In one experiment only 600 mg of this amino acid were allowed, an amount predicted by means of regression equations to be adequate for only one-half of the subjects. In another test, 700 mg were administered to subjects whose minimum requirements were estimated to be between 500 and 700 mg, and in the final test 950 mg of lysine exceeded the estimated requirement of every subject by at least 250 mg. No attempt was made to induce a severe deficiency or to provide a large excess of lysine.

The foods as served supplied 400 or 450 mg of lysine. They were supplemented during the initial 12-day adjustment period with 1,000 mg of lysine as the monohydrochloride, and subsequently with the quantity necessary to increase the total lysine intake to 600, 700 or 950 mg.

Measurement of nitrogen balance. Urinary nitrogen and creatinine were determined daily. Feces for 6-day intervals were pooled. Fecal nitrogen values of individual subjects differed significantly from each other, but values for the same subject did not differ between periods. Therefore, in calculating nitrogen balances, the mean fecal nitrogen of each individual for all experimental periods was used.

RESULTS AND DISCUSSION

Data pertaining to nitrogen retention of subjects who consumed constant quantities of lysine, nitrogen and calories for 30 consecutive days are presented in terms

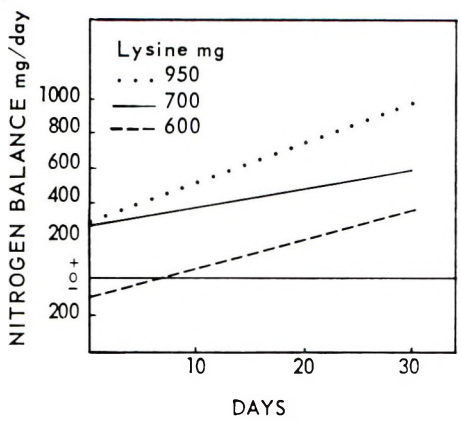

Fig. 1 Regression of nitrogen balance (Y) on time (X). The equations are: lysine 600 mg, $Y = 14.56X - 91$; lysine 700 mg, $Y = 10.74X + 288$; and lysine 950 mg, $Y = 22.15X + 294$.

of the entire experiment and of successive periods within it.

Regression of nitrogen balance on time. The slopes of the lines indicating the regression on time of mean daily nitrogen balances of three groups of individuals are depicted in figure 1. The regression equations related to intakes of 600, 700 and 950 mg of lysine were, respectively: 600 mg, $Y = 14.56\ X - 91$; 700 mg, $Y = 10.74X + 288$; and 950 mg, $Y = 22.15X + 294$, Y being the predicted nitrogen balance in milligrams and X being time in days. The regression of nitrogen balance on time was highly significant ($P < 0.01$) when lysine intake was 600 mg, and significant ($P < 0.05$) when it was 950 mg. It was not significant when 700 mg of lysine were consumed by 6 subjects for 30 days, but was significant ($P < 0.05$) when 4 of the same individuals continued for a total of 36 days.

Using these regression equations, one would predict that nitrogen retention of groups of subjects who consumed 600, 700 and 950 mg of lysine would improve by 440, 320 and 660 mg, respectively, between the beginning and end of a 30-day metabolism test. Such differences in nitrogen retention between the early and late phases of an experiment are of sufficient magnitude to require consideration in planning experiments and interpreting the results, whether dietary lysine is below, near or above the minimum requirement.

The slopes of the regressions of individual subjects did not differ significantly from each other within any group. None of the individual slopes differed from zero except that of subject ER when consuming 600 mg of lysine. Only one subject (WM) showed a downward trend during 30 days, and he improved sharply in a subsequent 6-day period. Since increments in nitrogen retention in 30 days that were predicted from individual equations varied by as much as 600 mg within a group, the importance of testing the effect of any dietary treatment on several subjects is emphasized.

The improvement of nitrogen balance with time in this series is in accord with observations of other investigators under distinctly different dietary conditions. After initiation of a diet containing minimal amounts of protein, nitrogen balances of men improved sharply for two weeks and then gradually for 5 weeks, finally reaching a plateau after 16 weeks (Horwitt et al., '56). When quantities of cereal protein near those required for equilibrium were fed, nitrogen balances of young women remained positive for 10 weeks with no tendency to decrease (Bricker et al., '49).

Effectiveness of different quantities of lysine in maintaining nitrogen equilibrium. One purpose of the present series was to examine critically the effectiveness in maintaining nitrogen equilibrium of the quantities of lysine that were reported earlier as minimum daily requirements for men and women. Therefore, nitrogen balances of individuals are expressed as means of 6-day experimental periods and of the entire experiment in table 2. Nitrogen intakes in all periods were between 9.0 and 9.1 gm. Mean fecal nitrogen values are indicated in a footnote to table 2. Mean creatinine values are shown in table 1. Body weights remained constant or increased slightly.

Nitrogen balances in the two initial 6-day adjustment periods were examined because the metabolic state in this interval might influence subsequent nitrogen retention. Rose ('57) observed that after the return of an essential amino acid whose omission had induced a strongly negative

TABLE 2

Mean daily nitrogen balances[1] in successive 6-day periods of subjects who consumed 9.0 gm of nitrogen and 600, 700 or 950 mg of lysine

600 mg lysine		700 mg lysine		950 mg lysine	
Subject	N balance	Subject	N balance	Subject	N balance
	mg		*mg*		*mg*
ER	−0.55	ER	0.24	WB	0.12
	−0.20		0.24		−0.03
	−0.13		−0.30		0.23
	0.08		0.25		0.47
	−0.08		0.58		0.44
Mean	−0.18 ± 0.23		0.20 ± 0.32		0.25 ± 0.21
CS	0.24	PG	0.37	GD	0.80
	0.15		0.66		1.01
	0.37		0.26		2.09
	0.57		0.24		1.74
	0.55		0.83		1.47
Mean	0.38 ± 0.18		0.47 ± 0.26		1.42 ± 0.52
JY	0.80	WM	0.52	JY	1.12
	−0.26		0.65		0.51
	0.38		0.27		0.65
	0.40		0.87		0.65
	0.59		0.19		1.14
Mean	0.38 ± 0.39		0.50 ± 0.28		0.81 ± 0.29
MZ	−0.26	EO	0.38	EO	0.36
	−0.37		0.23		0.31
	0.30		0.71		0.79
	0.01		0.81		0.81
	−0.04		0.70		1.05
Mean	−0.07 ± 0.26		0.57 ± 0.25		0.66 ± 0.32
		BS	0.25	RP	−0.34
			0.45		−0.19
			0.17		0.11
			0.77		0.10
			0.77		0.39
		Mean	0.48 ± 0.28		0.01 ± 0.29
		FS	0.25		
			0.39		
			0.59		
			0.60		
			0.66		
		Mean	0.50 ± 0.17		

[1] Balances positive unless so indicated. Mean fecal nitrogen (gm): ER 0.65, CS 0.60, JY 0.68, MZ 0.78, ER 0.65, PG 0.61, WM 0.92, EO 0.94, BS 0.59, FS 0.72, WB 0.78, GD 0.75, JY 0.72, EO 0.88, and RP 0.79.

balance the output of urinary nitrogen decreased progressively until equilibrium was re-established and then nitrogen was retained as the body compensated for the loss incurred during the period of deprivation. More recently, Hunscher ('61) emphasized the importance of reporting data obtained in adjustment as well as experimental periods. Several subjects in the present series maintained positive balance in both adjustment periods, others compensated during the second period for ni-

trogen lost in the first period, and only one subject (RP) failed to attain equilibrium during the adjustment interval. Thus, there was no reason to believe that nitrogen retention during the experiment was increased because of previous depletion of protein.

An intake of 600 mg of lysine was adequate throughout the experiment for only two of four subjects, since subjects ER and MZ did not attain equilibrium during the first two experimental periods. These findings are in complete agreement with earlier estimates of lysine requirements and also with estimates made from regression equations based on body weight, surface area and creatinine excretion of these individuals (Clark et al., '60). It would have been very unexpected if 600 mg of lysine had proven adequate for all. When 700 mg of lysine were provided, all subjects maintained positive balance except subject ER in one period. This amount of lysine therefore was satisfactory for men and women who weighed between 56 and 67 kg. Four of the 5 men who weighed from 64 to 76 kg and ingested 950 mg of lysine consistently maintained equilibrium or positive balance. The failure of subject RP to attain equilibrium in the first two periods cannot be explained, because the lysine intake exceeded his predicted requirement by 250 mg.

The findings in these three experiments are consistent with minimum lysine requirements estimated in short-term tests (Clark et al., '60). Attainment of equilibrium by most subjects at the beginning of the experiment and subsequent improvement by all individuals emphasize the usefulness of the estimated minimum requirements reported earlier, since the cereal-containing diet, essential amino acid supplements and total nitrogen intake were similar. When purified amino acids were ingested, Rose et al. ('55) observed that 800 mg or less of lysine met minimum requirements of all men tested. The regression equations developed for prediction of lysine requirements from body weight, surface area or creatinine excretion (Clark et al., '60) should be applicable to groups of subjects but cannot be expected to yield precise results for all individuals.

Variability of nitrogen balances in successive periods. The experimental plan permitted comparison of nitrogen balances of subjects in successive 6-day periods under constant experimental conditions. This subdivision of time within the experiment was selected because it was used when minimum lysine requirements of 10 men and 10 women were estimated (Clark et al., '60). Analysis of variance (Snedecor, '56) was conducted using mean nitrogen balance in each period of each individual within a group as an item of data, and then the new multiple range test (Duncan, '55) was applied.

Mean nitrogen balances in 5 successive 6-day periods of the group of subjects who consumed 600 mg of lysine were + 0.06, − 0.17, + 0.23, + 0.27 and + 0.26 gm; and of the group who consumed 700 mg of lysine they were in sequence, + 0.33, + 0.44, + 0.28, + 0.59 and + 0.62 gm. Mean balances in individual periods did not differ significantly from each other in either of these experiments, but they differed significantly ($P < 0.05$) when 950 mg were consumed, the values being + 0.41, + 0.32, + 0.77, + 0.75 and + 0.90 gm, respectively, in periods 1 to 5. Balances in periods 1 and 2 differed from that in period 5, and period 2 differed from 3 and 4. Differences such as these between periods under constant experimental conditions should be recognized. Dietary treatments should be so distributed within an experiment that effects attributable to position within the experiment are minimized.

Other investigators also have observed that nitrogen balances of subjects vary when experimental conditions do not change. Deviations in nitrogen retention in 7-day periods may be noted in the graphs published by Bricker et al. ('49) when women consumed a constant diet of cereals that supplied an amount of protein near the minimum need of each individual. Similarly, Johnston and McMillan ('52) reported that the amount of nitrogen retained by young women consuming 70 gm of protein varied widely from week to week for a particular subject but the means for 12 weeks were similar.

Reports in the literature relative to amino acid requirements and nitrogen re-

tention have included data based on periods of varying length. For this reason, the present experiment also was subdivided into intervals of 4, 8 or 10 days. Differences between highest and lowest balances of subjects who consumed 700 mg of lysine were 0.21 gm greater, on the average, when calculated for 4-day than for 6-day periods. Data representing as little as 4 days therefore would reflect the response to a particular dietary treatment less satisfactorily than a longer interval. On the other hand, expression of the data in terms of periods of either 8 or 10 days reduced the deviation between maximum and minimum values 0.16 gm below that noted in 6-day periods. Because of variations in urinary excretion from day to day, periods should be of sufficient length to provide clear-cut results.

Variability in nitrogen balances of different individuals. Nitrogen balances characteristic of certain individuals differed from others when data for all 6-day periods were considered. Differences between individuals were highly significant ($P < 0.01$) when 600 mg of lysine were provided, nitrogen balances of subjects CS and JY being higher than those of subjects ER and MZ (table 2). Differences were not detected when 700 mg of lysine were administered, but were highly significant ($P < 0.01$) when 950 mg of lysine were tested. Subject GD differed from all others in the group, and RP differed from all except WB who in turn differed from subjects EO and JY. Differences in metabolic responses and amino acid requirements of individuals have been observed in several laboratories. Since the individuals who comprise any group may influence the results, as many subjects as possible should be tested simultaneously.

SUMMARY

The influence on nitrogen retention of providing constant intakes of lysine and nitrogen during a 30-day interval was tested in three groups of human subjects. The experimental diet contained cereals, purified amino acids and diammonium citrate and supplied 9.0 gm of nitrogen daily. In different experiments, 600, 700 or 950 mg of lysine were provided. Earlier estimates of minimum lysine requirements of men and women which were based on short-term experiments were supported. Nitrogen retention improved with time whether 600, 700 or 950 mg of lysine were consumed. Variability in nitrogen balances of different subjects in successive periods of varying length has been discussed.

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Effects of Thiamine Deficiency and of Oxythiamine on Rat Tissue Transketolase[1]

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Following the demonstration that the addition of methylene blue to intact rat erythrocytes stimulated a recycling glucose oxidative pathway (Brin and Yonemoto, '58), it was shown that the activity of this metabolic system was markedly depressed in intact red cells from the thiamine-deficient rat (Brin et al., '58), and man (Wolfe et al., '58), and in intact erythrocytes obtained from rats treated with oxythiamine (Wolfe, '57). A hemolysate assay procedure was developed (Brin et al., '60) which was more suitable for routine work. Transketolase assays on hemolysates were shown to be specific and sensitive to thiamine deficiency (Brin et al., '60; Brin, '62). It was desirable, therefore, to reaffirm the effect of oxythiamine on hemolysate transketolase and to extend the observations to the transketolase activity of 8 other tissues of the rat.

EXPERIMENTAL PROCEDURE

Seventy-two female albino rats[2] weighing between 60 to 80 gm were divided into groups coded as follows: the code "C" denoted rats that were fed the purified diet with adequate thiamine. The percentage composition of the diet was glucose, 73; purified casein, 18;[3] corn oil, 4; salt mixture (Hegsted et al., '41), 4;[4] cod liver oil, USP, 1. The following vitamins were added in mg/kg of diet: thiamine·HCl, 4; riboflavin, 8; pyridoxine·HCl, 4; Ca pantothenate, 25; niacin, 40; choline chloride, 1,000. The code "D" denoted rats that were fed the purified diet devoid of added thiamine. The sign "+" indicated rats, that were treated with oxythiamine by daily intraperitoneal injection at a level of 100 μg/day. Rats that were not treated with oxythiamine were injected with an equal volume of 0.9% NaCl daily. The rats injected with oxythiamine were housed individually, whereas other rats were housed in groups of 6 in wire-bottom cages with food and water available ad libitum. All rats were weighed three times weekly for a period of two weeks. Rats from each coded group were killed at 4, 7, and 13 days after the initiation of the dietary regimen. At the time of sacrifice, blood was withdrawn by cardiac puncture from the ether-anesthetized animal. The rats were exsanguinated by decapitation and samples of spleen, skeletal muscle (femoral), heart, lung, liver, kidney, intestine (ileum), and brain (whole) were removed, wrapped in Parafilm[5] and frozen at $-20°C$ for periods of one to three months until analyzed for transketolase activity.

The preparation of, and transketolase assay of erythrocyte hemolysates were described previously (Brin et al., '60) except that the orcinol reagent was modified as heretofore described (Brown, '46). Tissue samples were weighed on a Roller-Smith torsion balance and were diluted with suitable volumes of buffer to yield the following dilutions: liver and kidney 1:50; brain, heart, muscle, lung, intestine, and spleen 1:10. For the transketolase assays, an homogenate aliquot of 0.5 ml/assay tube was used for all tissues with the

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[2] These rats were previously maintained with Big Red Animal Food produced by the Cooperative GLF Exchange, Inc., Ithaca, New York. The guaranteed analysis was, in per cent, crude protein, 24; crude fat, 5.0; and crude fiber 5.0.
[3] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.
[4] Obtained from Nutritional Biochemicals Corporation.
[5] American Can Company, Marathon Division, Menasha, Wisconsin.

TABLE 1

Relative effects of thiamine deficiency and the administration of oxythiamine (by injection), on transketolase activity in rat tissues,[1] after a 13-day experimental period

Tissue	Transketolase activity							
	C	C+	D	D+	C	C+	D	D+
	mg hexose/hour				*mg pentose/hour*			
Erythrocyte	1.8 ± 0.06[5]	0.86 ± 0.08[6]	0.56[4] (0.49–0.66)	0.23 ± 0.06[5]	2.3 ± 0.05	1.7 ± 0.05	1.6 (1.5–1.7)	1.2 ± 0.03
Liver	51 ± 2.5[5]	45 ± 2.5[5]	27[2] (26–28)	6.2[4] (5.3–7.0)	94 ± 2.3	80 ± 4.7	66 (64–68)	35 ± 1.7
Brain	8.1 ± 0.25[5]	7.7 ± 0.25[5]	8.4 ± 0.09[5]	7.9 ± 0.17[6]	14.3 ± 1.4	12.9 ± 0.42	14.4 ± 0.26	13.3 ± 0.66
Kidney	31 ± 2.3[5]	25 ± 2.8[5]	10 ± 0.98[5]	6.5 ± 0.67[5]	45 ± 3.7	45 ± 2.3	22 ± 2.0	17 ± 2.4
Heart	2.2 ± 0.05[5]	1.1[4] (0.8–1.3)	0.96[3] (0.41–1.5)	0.74 ± 0.25[3]	6.5 ± 0.17	5.7 (5.0–6.7)	5.0 (4.8–5.2)	4.2 ± 0.39
Muscle	0.92 ± 0.08[5]	0.79 ± 0.17[3]	0.77[4] (0.4–0.9)	0.70 ± 0.25[3]	6.3 ± 0.57	6.9 ± 0.48	6.4 (5.3–7.5)	6.0 ± 0.06
Lung	10 ± 0.44[5]	6.5 ± 0.2[5]	3.5[3] (2.5–5.2)	2.3[4] (1.9–2.7)	15.9 ± 0.42	11.8 ± 0.33	8.1 (7.3–9.8)	6.3 (5.7–6.9)
Intestine	14 ± 0.43[5]	11.2 ± 0.83[5]	5.0[4] (4.5–5.4)	3.3 ± 0.23[5]	18.7 ± 0.47	17.3 ± 0.66	10.0 (8.9–10.5)	7.4 ± 0.31
Spleen	14.5 ± 1.6[5]	13.8 ± 1.6[5]	5.8[2] (4.9–6.7)	3.7[3] (3.1–4.7)	18 ± 1.6	18 ± 2.0	9.0 (8.3–9.7)	6.9 (6.3–7.6)

[1] The data are expressed as mean ± SE. The number analyzed is shown in the superscript; where 4 or fewer were analyzed the mean and range is given. Transketolase activity for erythrocytes is calculated per milliliter of hemolysate, while for tissues it is calculated per gram of fresh tissue. The signs "C" and "D" denote the thiamine-adequate and the thiamine-deficient groups, respectively. The "+" denotes the administration of oxythiamine by injection at the level of 100 μg/day.

exception of liver. In the latter case 0.3 ml was used and the difference was made up with buffer. Both the disappearance of pentose and the formation of hexose were measured in all assays.

RESULTS

The weight gains of the rats fed the thiamine-deficient diet, and of the thiamine-adequate and thiamine-deficient rats that were treated with oxythiamine, were similar to those observed in the untreated thiamine-adequate group. The gross physical appearance and the behavior of the rats were normal during the 13-day experimental period.

Data to show the effects of thiamine deficiency, and of administering oxythiamine to thiamine-adequate or thiamine-deficient rats, for a period of 13 days, on the transketolase activity in erythrocytes and 8 tissues are presented in table 1. The variation in the number of transketolase assays per group was due to occasional deaths, insufficient tissue, or loss of samples during the analyses.

A 50-fold difference in the transketolase activity of the various tissues obtained from untreated rats was noted, with liver having the highest activity. The other tissues, when listed in the order of decreasing enzyme activity, were kidney, spleen, intestine, lung, brain, heart, erythrocyte and muscle.

Data similar to that presented in table 1 were obtained for erythrocytes and tis-

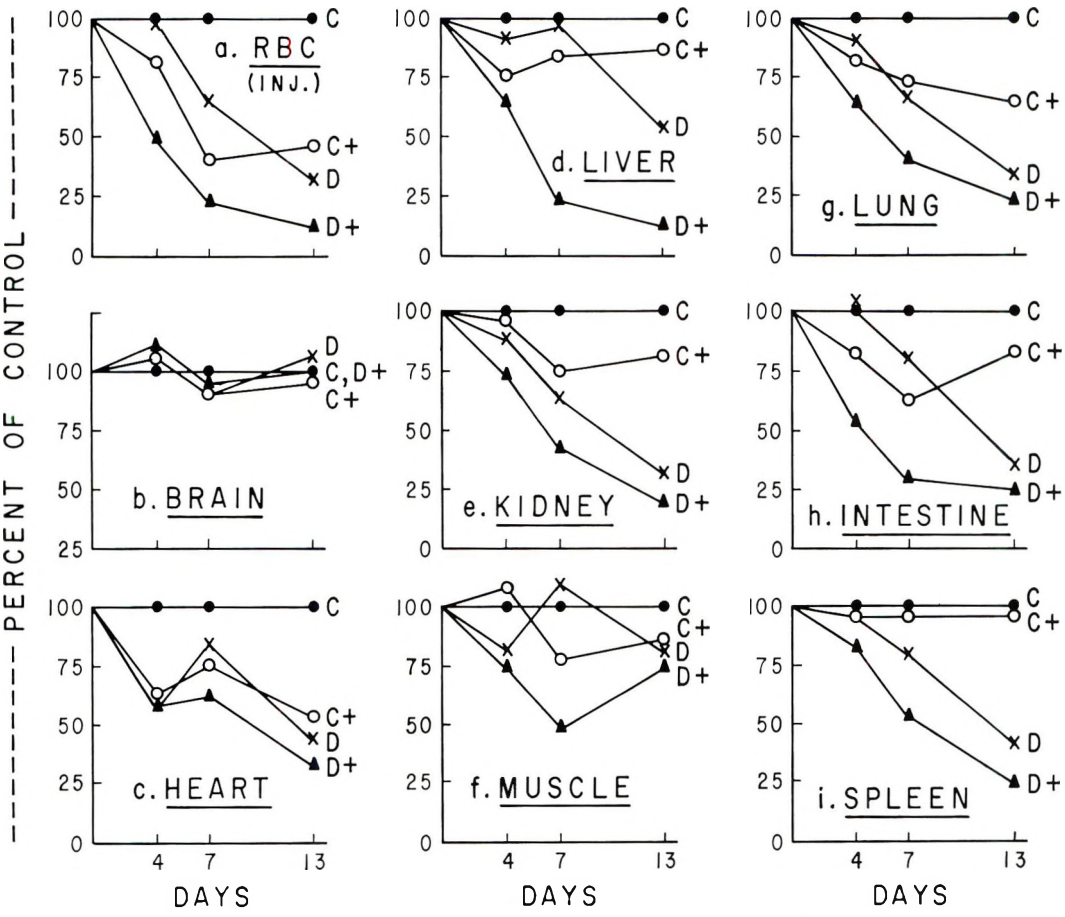

Fig. 1 The effects of injected oxythiamine (+) on the transketolase activity (formation of hexose) of 9 tissues in control (C) and thiamine-deficient (D) rats. Data are expressed as percentage of the control (C) group.

sues after 4 and 7 days of the experimental regimen. To present the material in a more unified fashion the data were recalculated in each case as a percentage of the "C" or untreated group. These values are presented graphically in figure 1.

By the fourth experimental day transketolase activity was decreased in heart from thiamine-deficient rats. At 7 days it was decreased in the erythrocytes, heart, kidney, lung, intestine and spleen. After 13 days it was depressed even more so in erythrocytes, heart, liver, kidney, muscle, lung, intestine, and spleen in thiamine-deficient animals. Transketolase activity in brain obtained from thiamine-deficient rats was unaffected after 13 experimental days.

The administration of oxythiamine to thiamine-adequate or thiamine-deficient rats for a 13-day period decreased the transketolase activity of erythrocytes, heart, liver, kidney, muscle, lung and intestine. The transketolase activity of spleen obtained from oxythiamine-treated, thiamine-adequate rats was unaffected at 13 days, whereas it was decreased in spleen obtained from thiamine-deficient rats. The transketolase activity of brain obtained from oxythiamine-treated, thiamine-adequate or thiamine-deficient rats was unaffected after 13 experimental days. Similar effects were observed when oxythiamine was administered by adding it to the diets at a level of 10 μg/gm diet.[6]

DISCUSSION

Previous studies on the thiamine content of rat heart, liver, kidney, and brain, as determined at intervals over a 4-week period of thiamine deficiency demonstrated that kidney, heart and liver thiamine decreased at a uniform rate with time as the deficiency progressed (Salcedo et al., '48). Normal levels of brain thiamine were maintained in these rats for the initial two weeks, after which it decreased sharply. The data presented in this report on the effects of thiamine deficiency on rat tissue transketolase activity correlate very well, therefore, with the previous observations on tissue thiamine.

Growth, appearance, and behavior were normal in thiamine-deficient rats and in thiamine-adequate and thiamine-deficient rats that were treated with oxythiamine for the 13-day experimental period. Yet, both thiamine deficiency, and the administration of oxythiamine to thiamine-adequate and thiamine-deficient rats resulted in decreased transketolase activity of 8 tissues obtained from these animals. This effect was evident in most tissue after only 7 days of treatment, and brain was the only tissue in which the transketolase activity was not decreased by either thiamine deficiency or by oxythiamine during the experimental period. The sustained transketolase activity in brain was considered noteworthy because of the late development of the neurological signs of thiamine deficiency in the rat. It was clearly evident then, that the transketolase activity of all the tissue studied, with the exception of brain, was markedly depressed in thiamine-deficient rats before growth was depressed and before any other clinical signs were noted. These studies therefore lend support to the belief that erythrocyte transketolase, an enzyme readily available from the host without detriment, is an important reflection of the state of thiamine adequacy of rat tissues, particularly in marginal deficiency (Brin, '62; Brin et al., '61a, b).

SUMMARY

1. Transketolase activity varied markedly in different rat tissues. When listed in the order of decreasing activity per gram of fresh tissue they were liver, kidney, spleen, intestine, lung, brain, heart, erythrocytes and muscle.

2. The transketolase activity of all of the rat tissues tested with the exception of brain was decreased by thiamine deficiency. In heart tissue this effect was evident after 4 experimental days, and in all tissues (but brain) by 7 days; the effect was greater in the affected tissues after 13 days of thiamine deficiency. In lung, erythrocytes, kidney, and intestine the enzyme was depressed to about one-third of normal activity; in spleen, heart and liver it was depressed to about one-half of normal activity, and in muscle it was depressed to about four-fifths of normal activity after 13 experimental days of thia-

[6] M. Brin, unpublished experiments.

mine deficiency. Brain transketolase was unaffected in these rats.

3. Oxythiamine, when administered daily at a level of 100 μg/rat for 13 days by intraperitoneal injection resulted in decreased transketolase activity in all the tissues tested with the exception of brain, whether administered to thiamine-adequate or to thiamine-deficient rats.

4. Neither thiamine deficiency nor the administration of oxythiamine to thiamine-adequate or thiamine-deficient rats resulted in adverse effects on growth, appearance, or behavior during the 13-day experimental period.

5. These studies lend support to the belief that the effect of thiamine deficiency on decreasing erythrocyte transketolase, a tissue readily available from the host without detriment, is a reflection of the thiamine adequacy of other tissues, particularly in a marginal deficiency.

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Effects of Levels of Certain Dietary Lipids on Plasma Cholesterol and Atherosclerosis in the Chick[1]

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There have been few reports of positive effects of dietary lipids upon cholesterol in the chicken. Stamler et al. ('59) reported only oleic, among the saturated and unsaturated acids, to have a hypocholesterolemic effect. Mone et al. ('59) and Kokatnur and Kummerow ('59) reported that plasma cholesterol in chicks is affected more by the level of dietary protein than by type or level of dietary fat. Others have since made similar observations. On the other hand, Marion et al. ('61) observed that corn oil had a greater hypocholesterolemic effect than did a dietary protein increase in cholesterol-fed birds. Hegsted et al. ('60) and Tennent et al. ('59) found that unsaturated fats lowered plasma cholesterol in cholesterol-fed birds.

Hypocholesterolemic effects have been observed with 10% of either ling cod liver oil or halibut liver oil in the diet (Wood and Biely, '60). A high vitamin A fraction from the ling cod liver oil was found to have the effect (Wood, '60) and this was attributed partly to the vitamin A present. Weitzel et al. ('56) had reported that high dietary levels of vitamin A and E reduced atheromatous lesions in old hens. Vitamin E is reported to be ineffective in the chick (Dam, '44; Stamler et al., '53; Weitzel et al., '56).

The purpose of the present work was to observe cholesterol levels in plasma and vascular tissues of chickens fed diets low in fat and high in saturated fat, with and without cholesterol and synthetic vitamins A and E.

MATERIALS AND METHODS

Two basal diets were used, one containing 20% of fat (high-fat) and the other 0.5% fat (low-fat). The percentage composition of the high-fat diet was: isolated soybean protein,[3] 32.33; glucose,[4] 31.27; fat, 20.00; mineral mix (Krautmann et al., '58, modified to include 0.00006% $Na_2MoO_4 \cdot 2H_2O$), 7.20; dried whey, 6.47; DL-methionine, 0.71; glycine, 0.39; vitamin premix, 1.63. The vitamin premix supplied for each 100 gm of diet: choline chloride, 258 mg; riboflavin, 1.13 mg; Ca pantothenate, 2.27 mg; niacin, 5.12 mg; inositol, 31 mg; pyridoxine·HCl, 1.73 mg; thiamine·HCl, 1.02 mg; folic acid, 0.48 mg; biotin, 0.04 mg; vitamin B_{12}, 1.28 μg; vitamin A, 517 IU; vitamin D_3, 291 ICU; menadione sodium bisulfite, 0.09 mg; vitamin E, 0.71 IU: penicillin, 0.85 mg. To maintain a constant ratio to total energy, the protein, mineral and vitamin levels were multiplied by the factor 0.773 in converting this diet to the low-fat diet. Glucose was then added to adjust the ration to 100%. Additions were made as follows to both basal diets at the expense of glucose: gelatine-coated crystalline vitamin A, 500,000 IU/gm;[5] vitamin E (*dl*-α-tocopherol);[6] sodium glycocholate,[7] and USP cholesterol.[8] The fats used were hydrogenated coconut oil[9] (HCO); stabilized yellow grease[10] (SYG); soybean oil[11] (SBO); and corn oil.[12]

Blood was obtained by heart puncture with a heparinized syringe. Plasma and aortic cholesterol were determined by the

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[1] Journal Paper no. 1895 of the Purdue University Agricultural Experiment Station, Purdue University, Lafayette, Indiana.
[2] Present address: Radioisotope Unit, V. A. Hospital, Madison, Wisconsin.
[3] Product no. 220, Archer-Daniels-Midland Company, Minneapolis, Minnesota.
[4] Cerelose, Corn Products Company, Argo, Illinois.
[5] Nutritional Biochemicals Corporation, Cleveland.
[6] Merck and Company, Inc., Rahway, New Jersey.
[7] See footnote 5.
[8] See footnote 5.
[9] Contribution of Procter and Gamble, Cincinnati; I.V. = 4.5.
[10] Darling and Company, Chicago; I.V. = 56.
[11] Contribution of Ralston Purina Company, Lafayette, Indiana; I.V. = 125.
[12] Corn Products Refining Company, Argo, Illinois.

method of Sperry and Webb ('50). Aortic lipids were extracted by the method of Folch et al. ('57). The resulting solutions were made to a suitable volume and aliquots were taken for the determination of total lipids and cholesterol. The solvent was removed under reduced pressure and the residue weighed as total lipid. The residue from a separate aliquot was taken up in 1:1 acetone:ethanol for the determination of cholesterol.

Statistical analyses were performed by the method of Newman ('39) as modified by Keuls ('52). Heterogeneity of variance was evidenced in both experiments, as a result of much larger variances in cholesterol-fed birds than in those not fed cholesterol. In experiment 1, to overcome this difficulty each variance of a difference from the average of the variances was calculated for that difference. Then the Newman-Keuls test was run on all possible pairs of means. In experiment 2, it was possible to obtain homogeneity of variance by dividing the treatments into two groups, cholesterol-fed and noncholesterol fed. Comparison of treatment means was made by the Newman-Keuls test within each group.

RESULTS AND DISCUSSION

Experiment 1. Sixteen groups of 15, one-day-old White Cross cockerels, were group-fed the diets as indicated in table 1, ad libitum. Eight one-day-old cockerels were found to have an average plasma cholesterol level of 550 mg/100 ml. Blood was obtained from 5 birds in each group after 4 weeks of treatment and from 5 different birds after 6 weeks of treatment. Plasma cholesterol data (table 1) for these two periods were combined and analyzed statistically.

Addition of cholesterol resulted in significant increases ($P < 0.05$) in plasma cholesterol levels in chicks receiving high-fat diets but not in those receiving low-fat diets. The addition of bile salt to the low-fat and 20% HCO diets resulted in nonsignificant further increases in plasma cholesterol.

Plasma cholesterol was significantly lowered ($P < 0.05$) in cholesterol-fed birds by high dietary levels of vitamin A. The ingestion of 42 IU/gm of diet (3,000 to 5,000 IU daily) was effective, and further effects from higher levels were small. Administration of 10,000 IU/day, subcutaneously, was less effective than lower levels given orally. Sodium glycocholate eliminated the hypocholesterolemic effect of the vitamin. The results suggest that the response is mediated through some effect on cholesterol absorption. Possibly the vitamin may exert its effect by interfering in some manner with bile acid metabolism, e.g., by forming an unabsorbable complex in the intestinal lumen. Such action could explain the effect on plasma

TABLE 1

Effects of various dietary lipids on plasma cholesterol levels in cockerels

Additions to various diets			Plasma cholesterol levels in chicks fed various fats[1]			
Cholesterol	Bile salt	Vitamin A	20% HCO	0.5% HCO	20% Corn oil	20% SBO
%	%	*IU/gm diet*	*mg/100 ml*		*mg/100 ml*	
0	0	0	143	133	—	—
1	0	0	485	147	354	293
0	0	167	109	—	—	—
1	0	167	147	—	277	—
1	0	83	175	—	—	—
1	0	42	262	—	—	—
1	0	100[2]	347	—	—	—
1	0	129	—	125	—	—
1	0.3[3]	0	623	198	—	—
1	0.3	167	676	—	—	—

[1] Averages of combined 4 and 6 week data; HCO, hydrogenated coconut oil; SBO, soybean oil.
[2] Vitamin A palmitate in ethyl palmitate 0.1 ml = 10,000 IU injected subcutaneously per day per bird.
[3] Reduced to 0.23% for the low fat diet.

cholesterol in chicks not fed cholesterol as well as in cholesterol-fed birds.

Corn oil[13] and soybean oil appeared to decrease plasma cholesterol even though this effect was not significant because of large variations within these groups.

Experiment 2. Nine groups of 25, one-day-old Arbor Acres cockerels, were group-fed the diets as indicated (table 2). Birds were weighed at zero, 3, 6, 10, 15 and 20 weeks. After 6 weeks, blood was obtained from 4 birds randomly selected from each group. Five other birds from each group were decapitated, after obtaining blood at the 10-, 15- and 20-week periods. The aortas were removed, thoroughly cleaned of all adhering materials, weighed, opened longitudinally and examined macroscopically for atherosclerosis. If present, the lesions were graded as to percentage of intimal area involved. Small sections of the ascending aorta were taken at 15 and 20 weeks and fixed in 10% formalin prior to histological preparation and examination. The remaining portions of the aorta were stored at $-20°C$ until further analyses could be performed.

The severity of atherosclerosis (table 2) was lowered by the addition of vitamin A or partial substitution of SBO in the HCO-cholesterol diet. Vitamin E alone showed no effect but appeared to diminish the effect of vitamin A. In aortas with macroscopic lesions, sudanophilic plaques were observed when frozen cross sections (20 μ) were prepared from the ascending aorta and stained with Sudan IV. No sudanophilic plaques were observed in any of the aortas of birds sacrificed at 15 weeks.

Body weight increases to 6 weeks of age (table 3) were significantly greater in birds receiving SBO or SYG ($P < 0.01$) than in birds receiving HCO diets. Group 4 receiving 20% of HCO, had significantly greater ($P < 0.01$) body weights than group 1, receiving 0.5% of HCO. The addition of cholesterol to the diet resulted in significantly lower body weights ($P < 0.01$) except when SBO was fed (group 9). Body weight trends observed at 6 weeks continued until the end of the experiment.

Plasma cholesterol data for the 4 periods were combined (table 3) for statistical treatment.

In cholesterol-fed chicks, plasma cholesterol levels were lowered significantly ($P < 0.01$) by either the addition of 100–167 IU of vitamin A/gm of diet (group 6) or the partial substitution of SBO for HCO (group 9). Since no significant effect of SBO was observed at the sixth week in either experiment 1 or 2, it appears that the response to SBO may be induced after this period. Separate statistical treatment of the data obtained at the 10-, 15- or 20-week periods consistently revealed a significant difference ($P < 0.01$) between the SBO (group 9) and HCO groups. Die-

[13] Mazola, Corn Products Company, Argo, Illinois.

TABLE 2

Atherosclerosis in chicks after 20 weeks of treatment (average values)

Group no.	Treatment			No. of chicks	No. with lesions	Intimal area involved		
	Cholesterol	Added vitamin[1]	Fat[2]			Thoracic aorta	Abdominal aorta	Brachio-cephalics
	%		%			%	%	%
1	0	0	0.5 HCO	5	0	0	0	0
2	0	0	20.0 SBO	5	0	0	0	0
3	0	0	20.0 SYG	5	0	0	0	0
4	0	0	20.0 HCO	5	0	0	0	0
5	1	0	20.0 HCO	7	6	38.5	24.3	44.4
6	1	A	20.0 HCO	7	5	1.4	1.0	4.5
7	1	E	20.0 HCO	5	5	31.0	21.0	51.0
8	1	A+E	20.0 HCO	9	5	17.2	10.0	29.0
9	1	0	10.0 SBO 10.0 HCO	8	3	0	1.2	0.1

[1] Vitamin A: 100 IU/gm diet, 0–6 weeks; 167 IU/gm diet, 7–20 weeks. Vitamin E: 0.15 mg/gm diet, 0–6 weeks; 0.25 mg/gm diet, 7–20 weeks.

[2] Hydrogenated coconut oil (HCO), I.V. = 4.5; soybean oil (SBO), I.V. = 125; stabilized yellow grease (SYG), I.V. = 56.

TABLE 3

Relationship of dietary lipids to growth, plasma cholesterol level and aortic lipids

Group no.	Body[1] wt	Total[2] plasma cholesterol	Aortic lipids[3]					
			10th Week			20th Week		
			Total lipids	Cholesterol		Total lipids	Cholesterol	
				Free	Total		Free	Total
	gm	*mg/100 ml*	%	%	%	%	%	%
1	815	123	1.95	0.13	0.14	1.56	0.15	0.17
2	1021	101	2.40	0.15	0.16	1.62	0.15	0.16
3	987	115	2.63	0.14	0.15	1.45	0.16	0.17
4	917	125	1.88	0.14	0.15	1.66	0.15	0.16
5	768	756	2.72	0.21	0.46	2.29	0.37	0.71
6	821	455	2.51	0.17	0.34	1.68	0.20	0.25
7	781	757	3.19	0.24	0.57	2.46	0.29	0.56
8	791	606	2.87	0.18	0.31	1.99	0.22	0.45
9	1015	348	2.73	0.16	0.23	1.80	0.17	0.20

[1] Averages of 6th week data.
[2] Averages of 6, 10, 15 and 20-week data.
[3] Average values.

tary vitamin E exerted no significant effect on plasma cholesterol.

In chicks receiving no dietary cholesterol, plasma cholesterol levels were not increased by high dietary levels of fat when compared with those fed the 0.5% HCO diet. However, the level in group 2 (20% SBO) was significantly lower ($P < 0.05$) than that in those groups receiving HCO.

In cholesterol-fed chicks significant correlation was found to exist between total plasma cholesterol and total aortic cholesterol (r was 0.52, 0.64 and 0.63 at 10, 15 and 20 weeks, respectively). Such a correlation did not exist in birds receiving no cholesterol (r was − 0.18, 0.11 and 0.24 for the same periods, respectively).

In all groups, although aortic cholesterol remained fairly constant throughout the tenth to twentieth week, other lipids decreased. Aortic cholesterol in birds with low plasma cholesterol levels was mainly in the free form. Aortas of birds fed cholesterol showed a marked increase in esterified cholesterol. In general, the severity of the atherosclerotic involvement of individual aortas was associated with an increasing esterified/free cholesterol ratio and a decreasing total lipid/total cholesterol ratio.

Those treatments that lowered plasma cholesterol decreased both the aortic cholesterol and the severity of atherosclerosis. Tennent et al. ('57) observed this relationship between plasma cholesterol and vascular lesions.

SUMMARY

Chicks fed a synthetic diet containing 20% of hydrogenated coconut oil (HCO) and 1% of cholesterol developed atherosclerotic lesions in 20 weeks. Cholesterol levels in the blood plasma and cholesterol esters in the aortic lipids were high in comparison with those of birds which received no dietary cholesterol. With a low-fat diet (0.5% HCO) plasma cholesterol was not elevated by cholesterol feeding.

Vitamin A (167 IU/gm of diet) inhibited sharply the vascular degeneration and the increase in plasma cholesterol and aortic ester cholesterol in chicks fed the high-fat diet. Vitamin E (0.25 mg of *dl*-α-tocopherol/gm of diet) showed no such effect. Bile salt (0.3%) in the diet nullified the effect of vitamin A. Substitution of soybean oil for half of the HCO inhibited the increase in plasma cholesterol and inhibited vascular changes and ester cholesterol accumulation similar to vitamin A.

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Effect of Severe Cold Stress on the Nitrogen Balance of Men Under Different Dietary Conditions[1]

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It is well known that in the adult organism a nitrogen equilibrium can be achieved and maintained at any level of protein intake above the protein minimum, provided there is no striking discrepancy between the daily caloric intake and the energy output. A restricted calorie supply as well as muscular work or exposure to cold can increase the urinary excretion of nitrogen. It has been observed repeatedly by many investigators that rats receiving a constant food intake excrete larger amounts of N at cold temperatures (Lathe et al., '49; Ingle et al., '53); You and his coworkers ('50) found that a cold environment (1.5°C) increased the urinary N excretion of rats, maintained with a daily food intake of 15 gm, from 400 mg to about 700 mg/day and that it was further increased to about 900 mg/day when the food intake was doubled. In these experiments, the metabolic rate was increased to at least twice that of the resting metabolic rate. No comparable data are available on men exposed to cold because the experiments have been carried out either on persons dressed in shorts at a temperature of 15 to 16°C (Iampietro et al., '57), or at severe cold but protected by Arctic clothing (Bass, '60). In either case, the increase of the metabolic rate was of the order of 25 to 50% above the basal metabolic rate (BMR) (Horvath et al., '47).

The purpose of the present investigations was to expose healthy, young men to cold stress which induces an almost continuous shivering with a concomitant increase of the metabolic rate to at least twice the BMR. It was hoped that under such circumstances, using 4 diets, a study of the N balance would yield information about the protein metabolism of men in the cold and its relationship to the overall increase in metabolic rate.

METHODS

Eighteen healthy, young men 19 to 29 years of age, with body weights ranging from 56 to 86 kg, and heights between 170 and 186 cm, served as subjects for these studies. A total of 39 experiments were carried out, of which 30 took place at a room temperature of 22°C. Nine subjects spent from 3 to 9 days and nights at 8°C continuously, wearing only cotton shorts, a pair of light wool socks and sneakers. For the sleeping period from 10:00 P.M. to 7:00 A.M., they were given a wool blanket. The N content of the diet, and that of the 24- or 48-hour urine and the 48-hour stool collections were analyzed by the micro-Kjeldahl procedure (Niederl, '42). The 48-hour stools were homogenized under continuous stirring with 50% sulfuric acid, brought up to 2,000 ml with water, and an aliquot was taken for N determination. Urine creatinine was determined according to the method described by Anker ('54).

The composition of the 4 test diets is shown in table 1. Diet 1 is the so-called normal diet, 3,000 Cal. with 72 gm of protein or 11.5 gm of protein N. Diet 2, with the same caloric value as diet 1, contained only about 4 gm of protein, or 0.5 gm of nitrogen. Diet 3 had about the same amount of protein as diet 1 (11.8 gm N) but the caloric intake was reduced by 50%. Diet 4 contained 1,500 Cal. and about 0.5 gm of protein N.

Prior to each experimental period, the subjects were fed diet 1 for several days. The experimental period was then started, the diet to be tested being fed for 10 days either with the subjects at 22°C, or during

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TABLE 1

Effect of dietary stresses on nitrogen balance in normal young men living at 22°C for 10 days

	Diet 1		Diet 2		Diet 3		Diet 4	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Protein (as gm N/day)	11.5	(11.2–12.0)	0.5	(0.3–0.7)	11.8	(11.0–12.6)	0.6	(0.5–0.8)
Fat, gm/day	94	(84–103)	105	(96–113)	64	(57–74)	70	(66–79)
CHO, gm/day	465	(436–497)	509	(497–521)	165	(157–174)	214	(198–223)
Cal./day	2987	(2951–3038)	3002	(2958–3067)	1523	(1430–1574)	1504	(1495–1510)
No. of subjects		7		7		6		7
Change in body wt, kg ± SD		− 0.4 ± 0.3		− 1.0 ± 1.2		− 2.0 ± 1.3		− 3.0 ± 0.8
Mean fluid intake, ml/day (a)		2,706 ± 435		2,900 ± 500		2,363 ± 311		2,396 ± 309
Mean urinary output, ml/day (b)		1,435 ± 402		1,439 ± 405		1,315 ± 397		1,139 ± 329
Difference: a − b ± SD		1,271 ± 190		1,461 ± 151		1,048 ± 159[1]		1,257 ± 158[1]
Cumulative N output, gm ± SD								
in urine		86.9 ± 12.5		36.6 ± 6.3		112.2 ± 8.9		50.9 ± 9.5
in feces		13.0 ± 3.9		9.4 ± 2.6		11.5 ± 2.1		8.5 ± 1.5
Cumulative N balance, gm ± SD		+ 7.8 ± 10.3		− 40.1 ± 6.2		− 6.9 ± 10.1		− 53.4 ± 10.1

[1] Significant difference, $0.02 < P < 0.05$.

cold exposure to 8°C in the climatic chamber for varying periods (3 to 9 days). After completion of the experimental period, diet 1 was again instituted for several days to obtain follow-up data. The activities of all the subjects during this study were limited so that their total energy expenditure at 22°C approximated 3,000 Cal./day. This was accomplished by two 30-minute periods of exercise daily on the bicycle ergometer at 600 m/kg/min. Distilled water was allowed ad libitum but the amount consumed was measured.

RESULTS

Studies at an ambient temperature of 22°C. Table 1 shows the mean changes of body weights during the 10-day experimental period at 22°C and the mean cumulative N balance data, as well as the daily fluid intake compared with the urinary output.

With diet 1, the mean N balance obtained in 7 experiments appeared to be slightly positive. However, under our experimental conditions the sensible and insensible water loss amounted to 1.5 liters/day (fluid intake plus about 250 ml of metabolic water less urinary output) and the nitrogen loss via sweating was not measured. The loss through the skin may be in the range of 0.1 to 0.3 gm/day even without visible sweating; exercise would increase this loss considerably (Rothman, '55; NAS, '56).

Diet 2 induced in 7 subjects an average accumulative N loss of 40 gm during the 10-day experiment.

With diet 3, the average N balance was slightly negative.

Diet 4 caused the greatest depletion of body proteins and exerted the heaviest dietary stress on the subjects. The mean accumulative N loss was 53.4 gm.

As was to be expected, the changes in N balance were dominated by the variations of the urinary output of nitrogen. A comparison of the fecal loss of nitrogen on protein-free diets (diets 2, 4) with the corresponding protein-containing diets (diets 1, 3) reveals that the daily excretion of food N is not more than 0.3 to 0.4 gm, which is well within the range of variations of the daily N intake. For this reason, the determination of fecal N seems

to offer no advantage over the assumption of a 1.3-gm/day loss of nitrogen via feces. Such an assumption appears to be not only justifiable, but it reduces the error in the calculation of the day by day N balance caused by an irregular bowel activity.

The difference between daily fluid intake and urinary output is somewhat greater with the protein-free diets (diet 2 and 4) than with the corresponding diets containing 72 gm of protein (diets 1 and 3). Although the difference between diets 3 and 4 is significant at the level of $0.02 < P < 0.05$, no conclusion can be drawn from these data since avenues of water loss other than urinary output were not measured.

Studies at an ambient temperature of 8°C. Diet 1. Figure 1 presents the daily course of the N balance, the changes in body weight as well as the daily fluid intake and urinary output with diet 1 at 22°C (fig. 1–A), compared with the data obtained for a subject fed the same diet but living in the climatic chamber at 8°C (fig. 1–B). With the basic diet (3,000 Cal., 72 gm of protein) at a room temperature of 22°C, this subject was in N balance and the body weight was kept fairly constant, despite the great variations in fluid intake and in urine volume (fig. 1 A). Similarly, the subject who participated in the cold experiment was also in N equilibrium and showed no change in body weight prior to entering the climatic chamber (fig. 1 B). However, during the first day in the cold, he lost about 1 kg, which was probably the result of the well-known cold diuresis. The fluid intake sharply decreased by about 750 ml and the volume of the urine increased by about the same amount. During the entire period in the cold, the difference between water intake and urine output was much less than at room temperature. This is probably due to two factors: (a) the loss of water via sweating is practically nil, and (b) the production of endogenous water due to

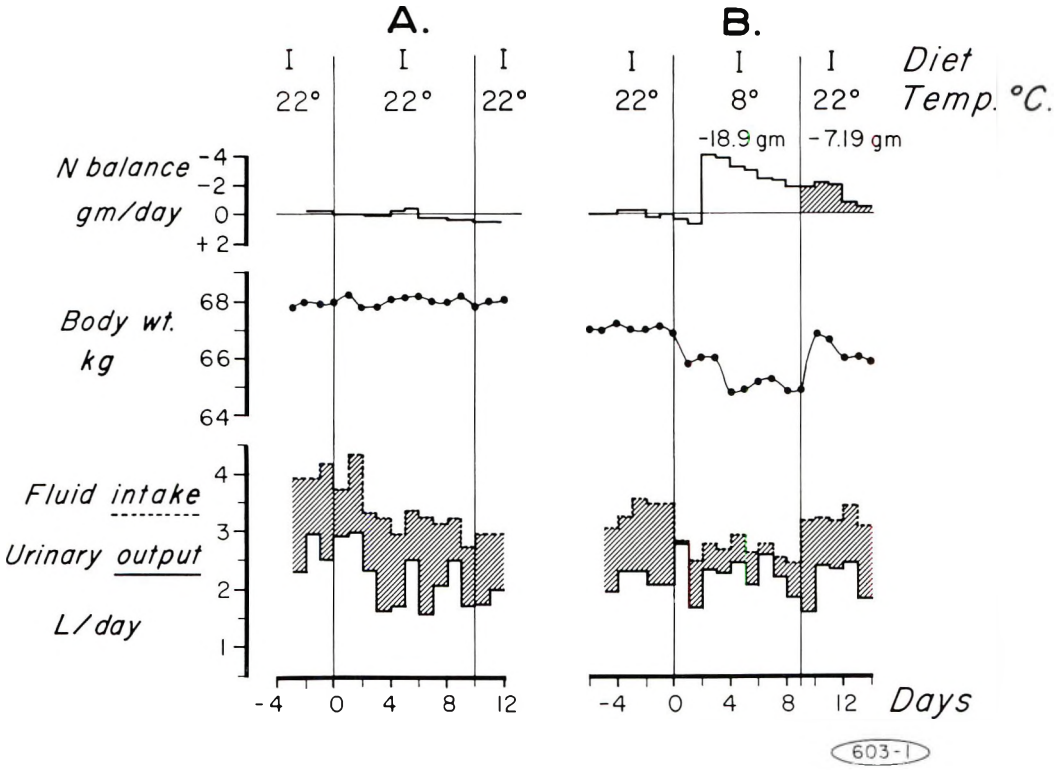

Fig. 1 Effect of 3,000 Cal. with 11.5 gm of protein N (diet 1) on the nitrogen balance at an ambient temperature of 22°C (1A) and at 8°C (1B).

the elevated metabolism is approximately doubled. The N balance in the first two days in cold did not show any change. This delayed onset of the negative N balance was rather unexpected because the shivering started within the first hour after entering the cold room and the metabolic rate was found more than doubled by the second day (figs. 2 B, 3 B).

The combined effect of depletion of body protein (a total of 18.9 gm of N), of an unknown amount of fat and the loss of water resulted in a 2-kg decrease of body weight in the subject living at 8°C and eating diet 1 for 9 days. Returning to a room temperature of 22°C, the first response was the repletion of body water, an increase of fluid intake, and a decrease of urinary output accompanied by a sharp increase in body weight. After leaving the cold room the negative N balance persisted at 22°C for 5 days, causing an additional N loss of about 7 gm. Both subjects who participated in this experiment showed almost an identical course of all these changes.

Diet 2: with diet 2 (3,000 Cal., 0.5 gm of N), the 10-day experimental period at 22°C caused a total N loss of 31.5 gm (fig. 2 A). The slight change in body weight, a loss of about 0.5 kg, was probably due to a change in the body water content rather than to the negative N balance. This is indicated by the rapid decrease of body weight on the first day of the protein-free diet and a similarly rapid increase on the first day of the follow-up period. During the recovery period with diet 1, the well-known repletion of body protein caused a positive balance. In 5 days, some 90% of the N loss was regained.

Two subjects spent three days in the cold room supplied with diet 2. Figure 2 B shows the observations on one of these subjects, which were quite similar in both subjects. The metabolic rate increased

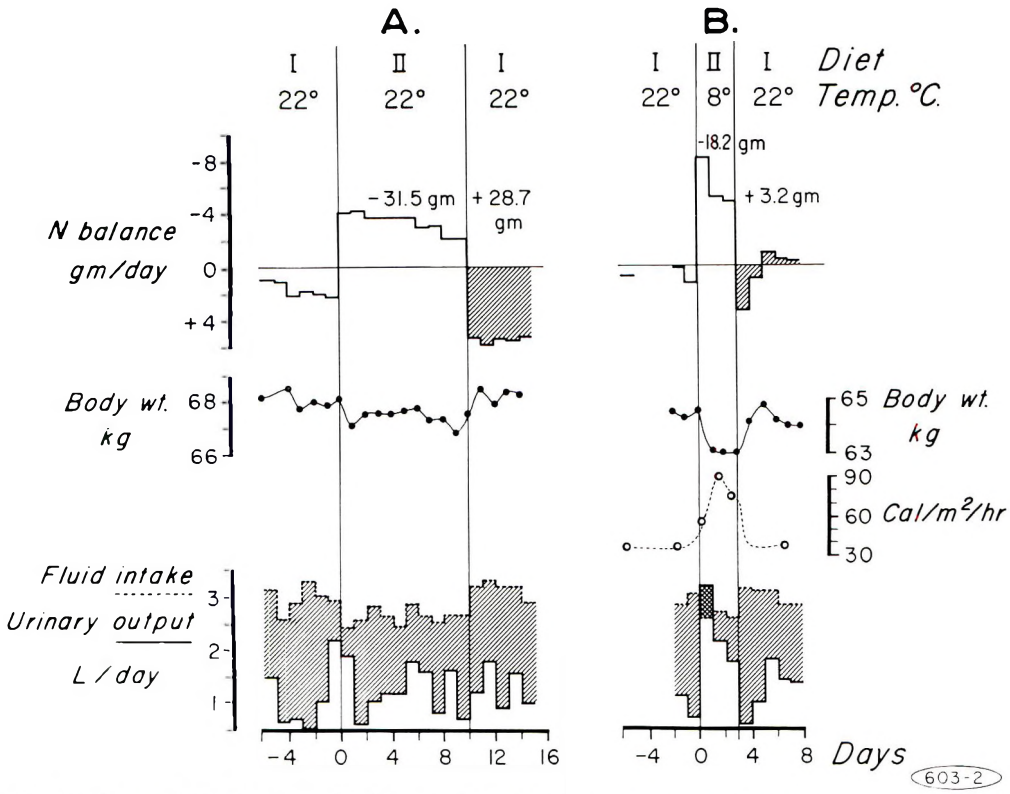

Fig. 2 Effect of 3,000 Cal. with 0.5 gm of protein N (diet 2) on the nitrogen balance at an ambient temperature of 22°C (2A) and at 8°C (2B).

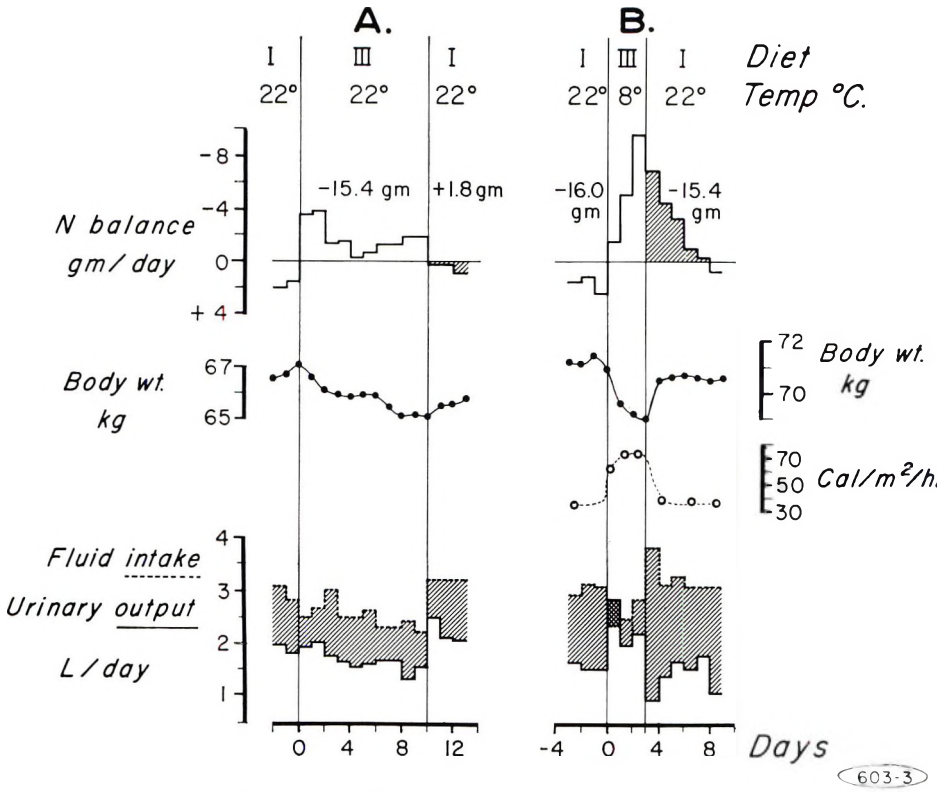

Fig. 3 Effect of 1,500 Cal. with 11.5 gm of protein N (diet 3) on the nitrogen balance at an ambient temperature of 22°C (3A) and at 8°C (3B).

from 37 Cal./m²/hour in about two hours in the cold room to 56 Cal./m²/hour, and the second day it reached a maximum value of 90 Cal./m²/hour. The body weight decreased in the first 24 hours by 1.5 kg, mainly due to the sudden decrease in the water content of the body. The urine volume exceeded the fluid intake by more than 600 ml. The repletion of the water occurred within the first day after leaving the cold room when the subjects were supplied with diet 1, causing a rapid increase in the body weight.

During the three days at 8°C, using diet 2, a total of 18.2 gm of N loss was measured. The most striking observation was the absence of a pronounced positive balance in the recovery period. Apart from the first 24-hour value after the subject was returned to diet 1 and a room temperature of 22°C, there was almost no repletion of protein despite an approximately 20-fold increase of protein intake at a metabolic rate of only 38 Cal./m²/hour.

Diet 3: This prolonged effect of cold exposure on the N balance was demonstrated even more strikingly when at the same time the caloric intake was reduced by 50%. Figure 3 shows the response of a subject to diet 3 (1,500 Cal. and 11.5 gm of protein-N) at 22°C (fig. 3 A) and at 8°C (fig. 3 B). This diet caused, during the 10-day experimental period at 22°C, a total of 15.4 gm of N loss. Since the difference, fluid intake less urine volume, was fairly constant throughout the experiment, and because of the gradual decrease of body weight of about 1.5 kg followed by a gradual weight gain during the follow-up period, the weight loss can probably be explained simply by the caloric deficit. After the subject was returned to diet 1 (3,000 Cal. with the same protein intake as diet 3), the N excretion decreased

immediately and the balance became slightly positive.

A comparable, 16-gm, N loss was induced in three days with diet 3 (fig. 3 B) at an ambient temperature of 8°C, whereas the metabolic rate increased from 36 Cal./m²/hour to 74 Cal./m²/hour. Here, too, as in all of our cold-room experiments, the cold diuresis caused a decrease of body weight by about 1.5 kg. On the first day in the cold, the amount of urine exceeded the fluid intake by about 500 ml. This 1.5 kg was regained on the first day at normal room temperature by increasing the fluid intake and reducing the urinary output so that the difference reached a value of almost 3 liters.

The N balance in the subjects fed diet 3 became negative on the first day in the cold and the negative balance increased continuously during the three days (fig. 3 B). This may be compared with the delayed onset of the negative balance with diet 1 (fig. 1 B), since the protein intake was the same in both cases.

An abrupt increase of the environmental temperature to 22°C, and a simultaneous doubling of the caloric intake (restoring the balance between caloric intake and caloric output), resulted only in a gradual decrease of the N loss. On the fourth day of the follow-up period, the balance was still negative despite the normal metabolic rate. During the three days in the cold and the 5 days' follow-up, the total cumulative loss of body nitrogen amounted to 31.4 gm with a very moderate weight loss of 0.5 kg. If we compare these figures with the 15.4-gm N loss accompanied by a weight loss of 1.5 kg with the same diet (diet 3) but at 22°C, the conclusion appears to be justified that the cold exposure specifically affected the protein metabolism.

Diet 4: Diet 4 (1,500 Cal. with 0.5 gm of protein-N) given to subjects living at

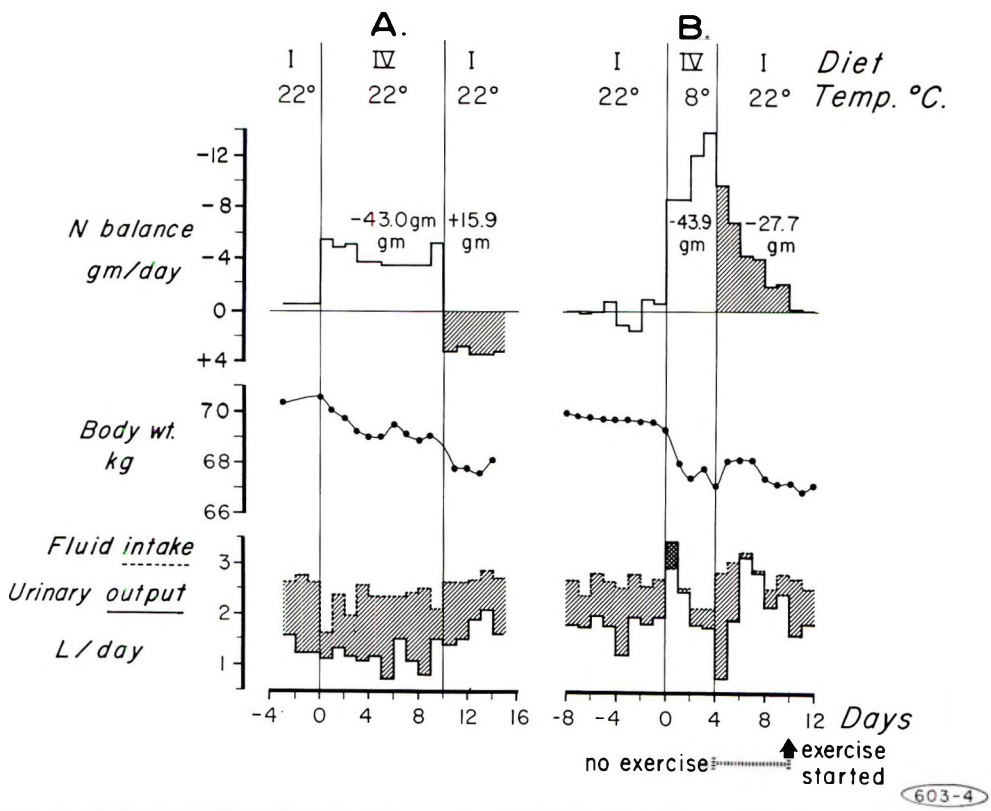

Fig. 4 Effect of 1,500 Cal. with 0.5 gm of protein N (diet 4) on the nitrogen balance at an ambient temperature of 22°C (4A) and at 8°C (4B).

8°C represented the most severe combined dietary and cold stresses. Here again, for comparison, two experiments were selected with almost the same degree of N loss. Figure 4 A shows that diet 4 induced in 10 days at 22°C approximately a 43-gm loss of body nitrogen. This was followed by a positive balance with a repletion of about 16 gm of protein N in 5 days with diet 1. The gradual decrease of the body weight, which continued even in the follow-up period and resulted in a 3-kg weight loss, can hardly be ascribed to any marked change in the water balance (except perhaps on the first day of the experimental diet). Four days in the cold room, using diet 4, induced a N loss of about 44 gm. There were, however, striking differences between the two experiments (figs. 4 A, B). In the cold, the N excretion increased gradually and the balance on the fourth day reached a value of − 14.2 gm (= a loss of 89 gm of body protein/day). During the follow-up period with diet 1 at 22°C, the N excretion decreased only gradually, leaving the balance still negative on the sixth day, and causing an additional loss of 27.7 gm of nitrogen. This occurred despite more than a 20-fold increase of the protein intake and a doubling of the caloric intake. Furthermore, the three subjects who were exposed to this most severe stress did not perform their daily bicycle ergometer exercise in the follow-up period because of intense vasodilatation and pain in the lower extremities. Their caloric requirement was therefore considerably below the 3,000 Cal. ingested. Nevertheless, none of the three subjects achieved a positive nitrogen balance during this period.

The body weight dropped in the first and second day in the cold by about 2 kg which was probably mainly due to the cold diuresis. At the end of the cold exposure, the body weight was 2.5 kg below the original value. After the subjects returned to diet 1 and to the room temperature of 22°C, the water retention caused only a transitory gain of about 1 kg. On the third and fourth days of the follow-up period, the volume of the urine was considerably increased and became practically equal to the fluid intake. This was probably the combined result of the lack of exercise (no sensible water loss) and the high urinary excretion of nitrogen.

If we compare the two experiments, the difference is striking (figs. 4 A, B). Whereas at room temperature, the consumption of diet 4 for 10 days and of diet 1 for 5 days, follow-up caused a total of 27.9 mg of N loss with a weight loss of about 3 kg, the 4-day cold exposure and the 5-day follow-up period resulted in a loss of 71.6 gm of body nitrogen accompanied by a weight loss of about 2.5 kg.

TABLE 2

Combined effect of dietary and cold stresses on the nitrogen balance

Subject no.	Experimental period			Recovery period Diet 1 (3000 Cal., 72 gm protein)	
		Diet	Cumulative N balance		Cumulative N balance
	days		*gm*	*days*	*gm*
		Temp 8°C			Temp 22°C
1	9	1 (3000 Cal., 72 gm protein)	−18.9	5	− 7.1
2	9	1 (3000 Cal., 72 gm protein)	−29.3	5	−11.3
3	3	2 (3000 Cal., 4 gm protein)	−21.4	5	+ 5.4
4	3	2 (3000 Cal., 4 gm protein)	−18.7	5	+ 3.2
5	3	3 (1500 Cal., 72 gm protein)	−10.5	5	− 8.0
6	3	3 (1500 Cal., 72 gm protein)	−16.4	5	−15.4
7	5	4 (1500 Cal., 4 gm protein)	−40.5	5	−12.4
8	5	4 (1500 Cal., 4 gm protein)	−45.7	5	− 4.1
9	4	4 (1500 Cal., 4 gm protein)	−43.9	5	−27.7
		Temp 22°C			Temp 22°C
7	6	4 (1500 Cal., 4 gm protein)	−23.6	5	+14.5
8	6	4 (1500 Cal., 4 gm protein)	−23.9	5	+20.1
9	6	4 (1500 Cal., 4 gm protein)	−26.2	5	+15.9

Here, too, the protein metabolism appeared to be specifically altered by the cold exposure.

One month later we had the opportunity to study the effect of diet 4 at 22°C on the same three subjects who participated in the cold-room experiments. During the 6 days they were supplied with this test diet, they lost 23 to 26 gm of nitrogen and during the 5 days' follow-up, they regained 15 to 20 gm of nitrogen (table 2, subjects nos. 7, 8, 9).

Table 2 summarizes the cumulative N balance data obtained with the 4 test diets during cold exposure (8°C) as well as the results of the 5 days' recovery with diet 1 at room temperature (22°C). Only in the case of diet 2 (3,000 Cal., 0.5 gm of nitrogen) consumed for three days in the cold, there was a slight repletion of body protein during the subsequent follow-up period with diet 1 at normal room temperature. In all other cases the negative N balance induced during the cold exposure continued during the follow-up period causing an additional loss of N.

DISCUSSION

These experiments showed an important difference between the depletion of body protein induced by dietary restriction, by exposure to severe cold, and by the combination of the two stresses. Whereas the recovery period following a diet insufficient either in protein or in both protein and total calories at normal room temperature was characterized by a rapid repletion of the protein stores of the body as indicated by the return to a positive N balance, no such repletion could be observed after a severe cold stress had been superimposed on the dietary stress. In the latter case, increased catabolism of body protein appeared to persist for 4 to 5 days after the cold exposure.

A possible explanation would be that in the cold, the twofold increased energy expenditure considerably exceeded the caloric intake and caused not only a depletion of body proteins but also a diminution of the glycogen stores. In the recovery period a refilling of the glycogen stores may require an intense neoglycogenesis from protein and this would result in a persisting negative nitrogen balance delaying the repletion of body protein. This could be the case especially when in the cold the caloric intake was also reduced to 1,500 Cal. (diets 3 and 4). Such a simple explanation, however, does not appear to be entirely satisfactory if we compare the effect of diet 4 for 10 days at 22°C with the effect of diet 2 for three days in the cold. The intake of 1,500 Cal. without protein (diet 4) at room temperature caused an N loss totaling 40 to 50 gm, with an average weight loss of 3 kg, whereas the 3,000-Cal. protein-free diet (diet 2) in the cold induced an N loss of 19 to 21 gm and a weight loss not more than 0.5 kg. The caloric intake in both cases was inadequate, yet in the former case the recovery period was characterized by a rapid repletion of body proteins, whereas after cold exposure no comparable response was observed. Similarly, also a comparison of diet 3 (1,500 Cal., 11.5 gm of protein N) at room temperature with diet 1 (3,000 Cal., 11.5 gm of protein N) in the cold room suggests that the energy balance is not the only factor responsible for the elevated and persisting protein catabolism. After supplying diet 3, the negative N balance disappeared as soon as the caloric intake was increased; on the other hand, with diet 1 it persisted for 5 days after the subjects were removed from the cold and the caloric output was markedly decreased.

The delayed onset of the negative N balance with diet 1 in the cold, its gradual increase with diet 3 and, above all, its persistence after the cold exposure, suggest that the cold stress induced a change in the hormonal balance which returned only gradually to the pre-exposure level.

Two groups of hormones are known to cause negative N balance: thyroid hormones and the glucocorticoids of the adrenal cortex. It is known that at least in animals, the activity of both endocrine organs is markedly increased in the cold (Hardy, '61). Their role in the cold-induced N excretion in rats was demonstrated by You et al. ('50). Whereas the urinary N output of normal rats was increased in cold (1.5°C) by about 300 mg/day, that of thyroidectomized animals was increased by only 92.7 mg/day and that of adrenalectomized rats by 81 mg/day.

In men exposed to cold, accelerated uptake and release of thyroid hormones were recently reported by Bass ('60). Watanabe and Yoshida ('56) found a significantly higher urinary excretion of 17 ketosteroids in the winter months compared with a very low output in the summer. It thus appears reasonable to look to the suggested hormonal imbalance as a possible explanation for the observed persistence of the negative nitrogen balance following severe cold exposure.

SUMMARY

Four groups of healthy young men consumed for 10-day periods at an ambient temperature of 22°C: 3,000 Cal., 72 gm of protein (diet 1); 3,000 Cal., 4 gm of protein (diet 2); 1,500 Cal., 72 gm of protein (diet 3) and 1,500 Cal., 4 gm of protein (diet 4), respectively. Diets 2 and 4 caused a marked loss, and diet 3 a moderate loss of body N. Changing to diet 1 during the follow-up period caused in every case a marked positive balance. The metabolic response to these 4 diets was investigated in 9 nude subjects living for three to nine days at an ambient temperature of 8°C. This cold stress induced almost constant shivering and a resting metabolic rate about twice the basal metabolic rate (BMR). A negative N balance was observed with diet 1, and the N loss characteristic for diets 2, 3, and 4 at 22°C was markedly increased at 8°C. Changing to diet 1 and a room temperature of 22°C during the follow-up period, during which time the BMR was normal, the negative N balance persisted for 4 to 6 days, despite an 18-fold increase of the protein intake (in the case of diets 2 and 4). It is suggested that an increased activity of the thyroid or the adrenal cortex, or both, may be responsible for this after-effect of cold exposure.

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Effectiveness of Urea and Ammonium Nitrogen for the Synthesis of Dispensable Amino Acids by the Chick[1]

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The utilization of nonprotein nitrogen by the chick has been investigated very little, although considerable research has been carried out on this aspect of the nutrition of other species. Lardy and Feldott ('49, '50) reported that rats fed diets containing various levels of 10 indispensable amino acids grew as well when the diets were supplemented with ammonium citrate as when they were supplemented with an isonitrogenous amount of dispensable amino acids. Rose et al. ('49) observed that ammonium salts, L-glutamic acid, glycine or urea markedly improved the growth of rats fed a diet containing only the indispensable amino acids. L-Glutamic acid and diammonium citrate were the most effective supplements. Glycine and urea were less effective but still markedly stimulated growth.

Birnbaum et al. ('57) and Rechcigl et al. ('57) have studied the relative effectiveness of various sources of nonprotein nitrogen for the synthesis of dispensable amino acids in the rat. Birnbaum et al. ('57) found that diets in which L-alanine, ammonium L-glutamate, L-glutamine, ammonium L-aspartate and L-proline were individually furnished as the sole sources of dispensable nitrogen supported most rapid growth. Ammonium acetate proved to be more effective than either urea or glycine. Rechcigl et al. ('57) observed that L-glutamic acid and a mixture of L-indispensable amino acids were the most effective supplements when judged by such criteria as growth response, feed efficiency and net nitrogen utilization. These were followed by alanine, aspartic acid, asparagine, proline, glutamine, diammonium citrate, urea, biuret, glycine and serine, all arranged in order of their effectiveness. Featherston et al. ('62) reported that chicks were able to utilize D- and excess L-indispensable amino acids as a source of nitrogen for dispensable amino acid synthesis for growth of 4 to 5 gm/day. In contrast, Stucki and Harper observed that an amount of indispensable amino acids well in excess of the requirement was not efficiently utilized by either chicks (Stucki and Harper, '61) or rats[4] as a substitute for dispensable amino acids.

Sullivan and Bird ('57) observed that additions of nitrogen in the form of urea or diammonium citrate to diets in which methionine and glycine had been replaced by their hydroxy analogues caused marked improvements in the growth and feed efficiency of chicks over that observed when the diets contained the hydroxy analogue alone.

This paper describes the results of studies in which chicks were fed diets containing only the indispensable amino acids supplemented with various sources of nonprotein nitrogen. The criteria used to assess the effectiveness of the various nitrogen sources included weight gain, feed efficiency, nitrogen retention and the concentrations of plasma amino acids.

EXPERIMENTAL

Day-old Cornish X White Rock chicks of both sexes were fed for one week a 10% isolated-soybean-protein diet to eliminate any possible carry-over effect of the absorbed yolk. The composition of the

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isolated-soybean-protein diet was identical to that described previously (Featherston et al., '62). At the end of one week chicks were weighed, wing-banded and separated into uniform groups. Chicks were housed in an electrically heated battery with raised wire-mesh floors. Feed was supplied ad libitum and fresh running water was available at all times.

In addition to the amino acid mixtures (table 1) and nonprotein nitrogen supplements, the diets used in these studies contained the following: (in per cent) soybean oil, 9.00; cellulose,[5] 3.00; mineral mixture (Briggs et al., '43), 6.00; vitamin mixture (Adkins et al., '62), 0.50; α-tocopheryl acetate (10 mg/gm), 0.10; vitamin D_3 (1500 ICU/gm), 0.08; vitamin A acetate (25,000 USP units/gm), 0.10; choline chloride (70% aqueous), 0.20; sodium bicarbonate, 1.00; magnesium trisilicate, 0.75; aluminum hydroxide, 0.25; and dextrin (moist cornstarch heated for two hours at 115°C in an autoclave, then dried and ground) to make the total mixture to 100%.

TABLE 1

Amino acid mixtures

Amino acid	1 L-AA[1] diet		1.25 L-AA diet	
	Form		Form	
		gm/kg		*gm/kg*
Arginine·HCl	L-	15.0	L-	18.75
Lysine·HCl	L-	12.5	L-	15.63
Histidine·HCl	L-	4.0	L-	5.00
Methionine	DL-	8.0	DL-	10.00
Tryptophan	L-	2.0	L-	2.50
Glycine	—	10.0	—	12.50
Phenylalanine	L-	7.0	L-	8.75
Leucine	L-	14.0	L-	17.50
Isoleucine	L-	6.0	L-	7.50
Threonine	L-	6.0	L-	7.50
Valine	L-	8.0	L-	10.00
Tyrosine	L-	7.0	L-	8.75

[1] AA indicates amino acid.

The 1L amino acid mixture (table 1) contained the L- forms of the indispensable amino acids, with the exception of DL-methionine, at levels which the National Research Council ('60) considers needed to satisfy the requirements of the chick. The 1.25 L-amino acid mixture contained 1.25 times the levels of the same amino acids used in the 1L amino acid mixture. DL-methionine was used in place of the L- form since the D- form is well utilized by the chick.

The chicks were weighed and the feed consumption determined at three-day intervals. The procedures for the nitrogen retention studies and plasma amino acid determinations were identical to those described previously (Featherston et al., '62). Blood samples were pooled for plasma amino acid determinations.

RESULTS AND DISCUSSION

Various levels of urea, diammonium citrate and dispensable amino acids were added to the 1 L-amino acid basal diet in the first experiment. The growth response, feed efficiency and nitrogen retention data of chicks fed the various diets are shown in table 2. The diets that contained 4 gm of urea or 15.1 gm of diammonium citrate/kg were isonitrogenous as were the diets containing 8 gm of urea, 30.2 gm of diammonium citrate or 34.6 gm of dispensable amino acids.

All additions of nitrogen, with the exception of the addition of 4 gm of urea, resulted in increased growth. Chicks in lots 4, 7 and 8 which were fed diets containing isonitrogenous amounts of urea, diammonium citrate and dispensable amino acids, respectively, gained weight at approximately the same rate. The same treatments which elicited growth responses in this experiment also elicited improvements in feed efficiency. Although the weight of chicks fed a diet containing 16 gm of urea/kg was no higher than that of chicks fed a diet containing 8 gm of urea/kg, an improvement in feed efficiency was noted. Chicks fed the diet containing dispensable amino acids gained weight more efficiently than chicks fed diets containing equal amounts of nitrogen from urea or diammonium citrate.

Nitrogen retention studies were conducted on chicks in lots 2, 4, 7 and 8. Diets fed to chicks in lots 4, 7 and 8 were isonitrogenous. Chicks fed urea and diammonium citrate retained a higher percentage of the ingested nitrogen than did the chicks fed the basal diet indicating that there was good utilization of the supplemental nitrogen. Chicks fed the diet sup-

[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Growth, feed efficiency and nitrogen retention data of chicks fed the 1 L-amino acid basal diet supplemented with different sources and levels of nitrogen

Lot	Supplement to basal diet[1]	Weight[2,3]	Gain[2]	Gm feed/gm gain	Nitrogen retained	Gm gain/gm nitrogen consumed
	gm/kg	*gm*	*gm*		%	
1	None	92.8 ± 9.6	34.8 ± 8.3	3.35	—	—
2	None	102.6 ± 7.6	44.4 ± 7.6	3.23	46.29	22.54
3	Urea, 4 gm	88.0 ± 5.2	29.4 ± 5.8	4.00	—	—
4	Urea, 8 gm	127.8 ± 6.1	69.6 ± 7.4	2.88	49.04	19.68
5	Urea, 16 gm	124.6 ± 7.1	66.0 ± 5.6	2.47	—	—
6	Diammonium citrate, 15.1 gm	121.0 ± 13.6	62.6 ± 13.1	2.76	—	—
7	Diammonium citrate, 30.2 gm	132.0 ± 12.9	73.6 ± 13.1	2.71	53.13	20.78
8	Dispensable amino acids,[4] 34.6 gm	132.4 ± 8.0	73.8 ± 7.9	2.59	55.77	22.02

[1] Diets fed to chicks in lots 4, 7 and 8 were isonitrogenous as were diets fed to chicks in lots 3 and 6.
[2] The mean ± SE of the mean of 5 chicks/diet; 18-day experimental period.
[3] Chicks in lots 4, 5, 7 and 8 were significantly ($P < 0.05$) heavier than those in lots 1 and 3 (Duncan's new multiple range test, '55).
[4] L-Glutamic acid, 15.38 gm; L-aspartic acid, 10.69 gm; L-proline, 3.67 gm; DL-serine, 2.95 gm; DL-alanine, 1.93 gm.

plemented with dispensable amino acids retained a higher percentage of nitrogen and also gained more weight per gram of nitrogen consumed than did chicks fed the diets containing urea, whereas chicks fed the diet containing diammonium citrate were intermediate in both respects.

The influence of these various diets on the concentration of amino acids in the plasma is shown in table 3. As was the case with growth rate in these chicks, all additions of nitrogen with the exception of 4 gm of urea, resulted in an increase in the concentration of total dispensable amino acids in the plasma as compared with that of chicks fed the basal diet. The low concentration of amino acids in the plasma of chicks fed the diet containing 4 gm of urea/kg probably resulted from low feed consumption, and this was probably also the factor responsible for the slow growth rate observed in these chicks. The concentrations of dispensable amino acids in the plasma increased as the level of nitrogen in the diet increased.

The concentration of indispensable amino acids in the plasma of chicks fed the basal diet was lower than that of chicks fed any diet containing an added source of nitrogen with the exception of chicks fed the diet containing 4 gm of urea/kg. This lower concentration of indispensable amino acids probably resulted from part of the nitrogen from the indispensable amino acids being used in the synthesis of dispensable amino acids since there was no other source of nitrogen available.

Thus it appeared that these various sources of nitrogen were equally effective in supporting growth of chicks fed diets containing the L-indispensable amino acids at required levels as set forth by the National Research Council ('60) and growing at rates of 4 to 5 gm/day.

It was, therefore, pertinent to determine whether similar results would be obtained when the levels of indispensable amino acids in the basal diet were increased. The 1.25 L-amino acid mixture (table 1) was used in the basal diet in this study. This L-mixture furnished 1.25 times the level of L-indispensable amino acids furnished by the 1 L-amino acid mixture. The dispensable amino acids were added in graded levels alone and with 1% of urea on the supposition that with lower levels of dispensable amino acids added urea might have a growth-promoting effect, whereas when the amount of dispensable amino acids added became adequate, urea would not be utilized. Additions of the dispensable amino acid mixture to supply nitrogen equivalent to 25, 50 and 100% of that supplied by the L-indispensable amino acids are signified by 25, 50 and 100% Disp-AA. The dispensable amino acid mix-

TABLE 3

Influence of diet on concentrations of plasma amino acids of chicks fed the 1 L-amino acid diet with various nitrogen supplements

	Amino acid concentrations Supplement to basal diet (gm/kg)						
	None	Urea			Diammonium citrate		Dispensable amino acids
		4 gm	8 gm	16 gm	15.1 gm	30.2 gm	34.6 gm
	μmole amino acids/ml plasma				*μmole amino acids/ml plasma*		
Glutamic acid	0.11	0.08	0.12	0.16	0.14	0.15	0.25
Glutamine + asparagine	0.16	0.10	0.21	0.44	0.30	0.48	0.39
Alanine	0.13	0.11	0.19	0.08	0.16	0.12	0.21
Proline	0.14	0.11	0.17	0.08	0.09	0.09	0.25
Hydroxyproline	0.03	0.06	0.09	0.07	0.05	0.05	0.10
	0.57	0.46	0.78	0.83	0.74	0.89	1.20
Serine	0.69	0.47	0.42	0.65	0.64	0.54	0.68
Tyrosine	0.26	0.25	0.25	0.27	0.45	0.25	0.30
	0.95	0.72	0.67	0.92	1.09	0.79	0.98
Threonine	0.44	0.34	0.37	0.51	0.50	0.43	0.39
Glycine	0.93	0.69	1.06	1.02	1.02	1.08	1.02
Lysine	0.22	0.12	0.47	0.27	0.49	0.34	0.22
Valine	0.18	0.14	0.19	0.20	0.24	0.15	0.32
Leucine + isoleucine	0.19	0.14	0.24	0.37	0.12	0.13	0.29
Phenylalanine	0.13	0.18	0.23	0.10	0.29	0.07	0.20
Methionine	0.04	—	—	0.10	—	0.07	—
	2.13	1.61	2.56	2.57	2.66	2.27	2.44
Total	3.65	2.79	4.01	4.32	4.49	3.95	4.62

ture was composed of L-glutamic acid, L-aspartic acid, L-proline, DL-serine and DL-alanine in the same relative proportions as they are found in soybean oil meal. One per cent of urea supplied approximately the same amount of nitrogen as supplied by the 25% Disp-AA treatment.

The results of this study are shown in table 4. The growth of chicks fed the basal diet supplemented with urea was approximately the same as that observed in the previous study. The growth of chicks fed the basal diet, with increased levels of indispensable amino acids, was better than that previously observed with the 1 L-diet, and was equal to that of chicks fed the urea supplemented diets. Chicks fed any of the diets containing dispensable amino acids weighed significantly more than chicks fed the basal diet or the diet supplemented with urea. The addition of 1% of urea to diets containing dispensable amino acids did not result in significant increases in weight. Growth of the chicks receiving the 50% Disp-AA was greatest. Feed efficiency was also improved as a result of dispensable amino acid supplementation, and increased as the level of dispensable amino acids increased.

The results of the nitrogen retention studies support the conclusions drawn from weight gain and feed efficiency data. The added nitrogen from urea was not retained or utilized as well as the added nitrogen from the dispensable amino acids. The highest percentage of nitrogen retention and gain per gram of nitrogen consumed resulted from the 25% Disp-AA treatment. The addition of higher levels of dispensable amino acids resulted in stepwise reductions in the percentage of nitrogen retained and gain per gram of nitrogen consumed. For the most part, the addition of 1% of urea to diets containing dispensable amino acids also resulted in decreases in the percentage of nitrogen retained and in gain per gram of nitrogen consumed.

The results of studies of the influence of the various diets fed in this experiment on the concentration of amino acids in the plasma of chicks are shown in table 5.

TABLE 4

Growth, feed efficiency and nitrogen retention data of chicks fed the 1.25 L-amino acid diet with various nitrogen supplements

	Supplement to 1.25 L-amino acid diet[1]	Weight[2,3]	Gain[2]	Gm feed/gm gain	Nitrogen retained	Gm gain/gm nitrogen consumed
		gm	*gm*		%	
1	None	122.8 ± 5.8	58.8 ± 4.7	2.14	59.42	25.92
2	1% Urea	118.8 ± 10.3	53.4 ± 9.1	2.36	49.11	19.08
3	25% Disp-AA	167.2 ± 10.9	102.8 ± 10.6	1.71	70.03	26.43
4	25% Disp-AA + 1% urea	165.6 ± 11.7	101.8 ± 11.6	1.83	58.62	19.63
5	50% Disp-AA	179.4 ± 13.4	115.4 ± 13.0	1.61	64.11	22.61
6	50% Disp-AA + 1% urea	176.8 ± 21.0	112.2 ± 21.6	1.62	54.99	18.77
7	100% Disp-AA	167.4 ± 12.7	103.4 ± 10.6	1.50	53.25	17.36
8	100% Disp-AA + 1% urea	179.4 ± 9.1	115.8 ± 10.8	1.50	53.64	15.95

[1] Twenty-five, 50 and 100% Disp-AA signifies additions of the dispensable amino acid mixture to supply nitrogen equivalent to 25, 50 and 100% of that supplied by the L-indispensable amino acids.
[2] The mean ± SE of the mean of 5 chicks/diet, 13-day experimental period.
[3] Chicks fed the diets containing dispensable amino acids were significantly heavier ($P < 0.05$) than chicks fed diets without dispensable amino acids (Duncan's new multiple range test, '55).

While the addition of urea to the 1.25 L-basal diet did not result in any increased growth, the concentrations of dispensable amino acids in the plasma of chicks fed diets supplemented only with urea were higher than those in the plasma of chicks fed the basal diet. When urea was added to the 25 and 100% Disp-AA diets, but not the 50% Disp-AA diet, the concentrations of plasma dispensable amino acids also increased. The concentrations of plasma dispensable amino acids in chicks fed the 50% Disp-AA diet were higher than those of chicks fed the 25% Disp-AA diet; however, the concentrations in plasma of chicks fed the 100% Disp-AA diet were lower, with one exception, than those of chicks fed the 50% Disp-AA diet. The plasma indispensable amino acids were observed to follow this same trend. A possible explanation for this observation is that the ratio of dispensable to indispensable amino acids in the 100% Disp-AA diet was not optimal and thus exerted a detrimental effect on food consumption.

The concentration of indispensable amino acids in the plasma of chicks fed the basal diet was again lower than that observed in chicks fed other diets, presumably owing to part of the nitrogen from the indispensable amino acids being used in the synthesis of dispensable amino acids. The plasma concentration of tyrosine decreased as the concentration of dispensable amino acids in the diet increased. The reason for this decrease is unknown.

Some of the results of this experiment were quite unexpected and made interpretation of the data difficult. Chicks fed the 1.25 L-basal diet gained about 4.5 gm/day, which is about double that observed for chicks fed the 1 L-basal diet and equal to that observed for chicks fed the 1.25 L-basal diet supplemented with urea. The increased growth of chicks fed the 1.25 L-basal diet suggests that the L-indispensable amino acids in this diet were present in sufficient excess so that a portion of them could be used as a source of nitrogen for the synthesis of dispensable amino acids and yet still leave enough to permit growth of this magnitude. Under these conditions, the extra nitrogen from urea was utilized only slightly if at all. The increased growth of chicks fed the 1.25 L-basal diet supplemented with dispensable amino acids as compared with that of chicks in the previous experiment which were fed the 1 L-diet supplemented with dispensable amino acids no doubt results from the increased amounts of indispensable amino acids available for tissue protein synthesis. Dispensable amino acids were superior to urea for growth of the magnitude observed in this experiment (8 to 9 gm/day).

TABLE 5

Influence of diet on concentrations of plasma amino acids of chicks fed the 1.25 L-amino acid diet with various nitrogen supplements

	Amino acid concentrations							
	Supplement to 1.25 L-AA diet							
	None	1% Urea	25% Disp-AA	25% Disp-AA + 1% urea	50% Disp-AA	50% Disp-AA + 1% urea	100% Disp-AA	100% Disp-AA + 1% urea
	μmole amino acids/ml plasma				*μmole amino acids/ml plasma*			
Glutamic acid	0.12	0.16	0.17	0.21	0.37	0.33	0.40	0.48
Glutamine + asparagine	0.23	0.30	0.27	0.66	0.89	0.71	0.56	0.83
Alanine	0.15	0.30	0.39	0.52	0.73	0.62	0.72	0.85
Proline	0.25	0.31	0.36	0.38	0.53	0.45	0.58	0.48
Hydroxyproline	0.13	0.10	0.10	0.16	0.18	0.17	0.11	0.14
	0.88	1.17	1.29	1.93	2.70	2.28	2.37	2.78
Serine	0.63	0.86	0.77	0.80	0.86	0.67	0.55	0.71
Tyrosine	0.67	0.69	0.55	0.55	0.35	0.28	0.11	0.19
Cystine	0.09	0.11	0.11	0.09	0.11	0.07	0.04	0.07
	1.39	1.66	1.43	1.44	1.32	1.02	0.70	0.97
Threonine	0.30	0.65	0.56	0.66	0.58	0.59	0.40	0.93
Glycine	1.10	1.11	1.09	1.24	1.04	1.06	0.63	0.70
Lysine	0.39	0.46	0.46	0.57	0.55	0.60	0.37	0.41
Valine	0.22	0.22	0.22	0.28	0.29	0.27	0.23	0.31
Leucine + isoleucine	0.18	0.20	0.21	0.27	0.32	0.32	0.29	0.25
Phenylalanine	0.29	0.31	0.33	0.26	0.26	0.28	0.19	0.19
Methionine	0.22	0.09	0.20	0.18	0.12	0.15	—	—
	2.70	3.04	3.07	3.46	3.16	3.27	2.11	2.79
Total	4.97	5.87	5.79	6.83	7.18	6.57	5.18	6.54

To make a critical evaluation of the utilization of these various sources of nitrogen and to study the relative effectiveness of dispensable amino acids and urea, a basal diet had to be used which did not supply an excess of indispensable amino acids. Therefore, in the third experiment the 1 L-basal diet was used with the same treatments studied in the second experiment.

Growth and feed efficiency data of chicks in this experiment are shown in table 6. The gains of chicks in this experiment were quite similar to those observed in the first experiment in which the 1 L-basal diet was also fed. As was the case in the first experiment, chicks fed the 1 L-basal diet in this experiment gained about 2 gm/day and chicks fed the basal diet supplemented with urea gained about twice as much, indicating that this source of extra nitrogen was being utilized under the existing conditions. Also, chicks fed two of the three diets supplemented with dispensable amino acids gained about the same amount as those fed the diet supplemented with urea as was the case in the first experiment. Whether the greater gains exhibited by chicks fed the 25% Disp-AA treatment was a real difference or was due to some unknown circumstance is not known. The addition of 1% of urea along with dispensable amino acids resulted in improvements in growth when the urea was added along with the 50 and 100% Disp-AA supplements. The response from the urea was less when added with the 100% Disp-AA supplement than when it was added with the 50% Disp-AA supplement which is what one would expect. The addition of 1% urea with the 25% Disp-AA supplement resulted in only a slight increase in growth. One would have expected the growth of chicks fed this level of dispensable amino acids to be improved most from urea supplementation. All treatments resulted in improvements in feed efficiency as compared to that of chicks fed the basal diet.

The results of studies on the influence of the various diets used in this experiment on the concentrations of plasma amino

TABLE 6

Growth and feed efficiency data of chicks fed the 1 L-amino acid diet with various nitrogen supplements

	Supplement to 1 L-amino acid diet[1]	Weight[2,3]	Gain[2]	Gm feed/gm gain
		gm	*gm*	
1	None	83.8 ± 7.9	27.8 ± 7.8	3.18
2	1% Urea	106.2 ± 8.6	50.2 ± 6.9	2.47
3	25% Disp-AA	147.6 ± 8.2	90.8 ± 8.1	1.72
4	25% Disp-AA + 1% urea	148.6 ± 10.1	93.0 ± 9.4	1.95
5	50% Disp-AA	101.2 ± 10.3	44.0 ± 10.1	2.47
6	50% Disp-AA + 1% urea	144.2 ± 19.5	88.2 ± 19.5	1.83
7	100% Disp-AA	107.2 ± 12.2	51.2 ± 11.7	2.16
8	100% Disp-AA + 1% urea	120.8 ± 8.7	64.8 ± 7.9	2.23

[1] Twenty-five, 50 and 100% Disp-AA signifies additions of the dispensable amino acid mixture to supply nitrogen equivalent to 25, 50 and 100% of that supplied by the L-indispensable amino acids.
[2] The mean ± SE of the mean of 5 chicks/diet; 15-day experimental period.
[3] Chicks fed diets 3, 4 and 6 were significantly ($P < 0.05$) heavier than chicks fed all other diets with the exception of diet 8 (Duncan's new multiple range test, '55).

TABLE 7

Influence of diet on concentrations of plasma amino acids of chicks fed the 1 L-amino acid diet with various nitrogen supplements

	Amino acid concentrations							
	Supplement to 1 L-AA diet							
	None	1% Urea	25% Disp-AA	25% Disp-AA + 1% urea	50% Disp-AA	50% Disp-AA + 1% urea	100% Disp-AA	100% Disp-AA + 1% urea
	μmole amino acids/ml plasma				*μmole amino acids/ml plasma*			
Glutamic acid	0.09	0.25	0.14	0.20	0.13	0.08	0.31	0.69
Glutamine + asparagine	0.18	0.62	0.84	0.72	0.75	0.47	0.54	0.55
Alanine	0.19	0.19	0.42	0.37	0.36	0.58	0.69	0.70
Proline	0.21	0.23	0.34	0.29	0.37	0.30	0.70	0.56
Hydroxyproline	0.06	0.10	0.11	0.13	0.12	0.08	0.10	0.11
	0.73	1.39	1.85	1.71	1.73	1.51	2.34	2.61
Serine	0.24	0.43	0.70	0.42	0.57	0.46	0.69	0.58
Tyrosine	0.37	0.42	0.42	0.36	0.17	0.23	0.19	0.20
Cystine	0.04	0.05	0.09	0.04	0.07	—	0.04	0.04
	0.65	0.90	1.21	0.82	0.81	0.69	0.92	0.82
Threonine	0.70	0.48	0.73	0.68	0.39	0.18	0.50	0.75
Glycine	0.79	0.84	1.28	0.93	0.63	0.80	0.75	0.79
Lysine	0.37	0.24	0.36	0.34	0.20	0.48	0.20	0.38
Valine	0.19	0.21	0.33	0.22	0.19	0.22	0.24	0.29
Leucine + isoleucine	0.15	0.18	0.27	0.15	0.19	0.23	0.20	0.36
Phenylalanine	0.21	0.21	0.23	0.20	0.18	0.18	0.16	0.19
Methionine	0.10	0.10	0.22	0.10	0.15	0.12	0.08	—
	2.51	2.26	3.42	2.62	1.93	2.21	2.13	2.76
Total	3.89	4.55	6.48	5.15	4.47	4.41	5.39	6.19

acids are shown in table 7. Although there was considerable variation among the individual values for the plasma amino acids in birds fed the various diets, some general observations can be made concerning the effect of these diets on the plasma amino acid concentrations. Chicks fed the diet containing urea alone had higher plasma concentrations of dispensable amino acids than did chicks fed the basal diet; however, none of the concentrations of dispensable amino acids in plasma of these chicks were as high as those of chicks receiving any of the diets containing dispensable amino acids. As in the preceding experiment, the concentrations of tyrosine

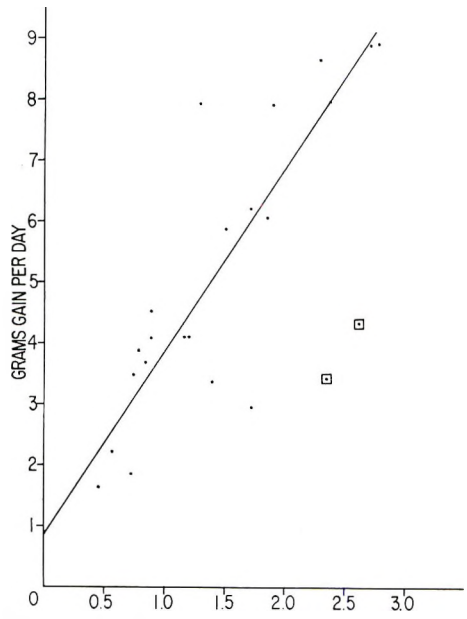

Fig. 1 Daily gain plotted against the dispensable amino acid concentration of chick plasma; ⊡ indicates values of chicks fed high levels of dispensable amino acids in the 1 L-amino acid diet.

in the plasma of chicks were observed to decrease as the level of dispensable amino acids in the diet increased even though the same amount of tyrosine was present in all diets.

These studies indicate that urea or diammonium citrate permitted gains in weight of chicks of similar magnitude (4 to 5 gm/day), although possibly not as efficiently, as obtained when dispensable amino acids were added to a diet containing only the indispensable amino acids at levels which the National Research Council ('60) considers to be needed to satisfy the requirement of the chick. When the levels of indispensable amino acids in the basal diet were increased so that more rapid growth was possible, these sources of extra nitrogen were not as effective as the intact dispensable amino acids. These findings are in agreement with those of Luckey et al. ('47) and Stucki and Harper ('61) who observed that dispensable as well as indispensable amino acids must be included in the diet for maximal growth of chicks. Similar conclusions have been obtained by numerous workers using the rat as an experimental animal.

A significant ($P < 0.01$) positive correlation was observed between the concentrations of dispensable amino acids of the plasma and the rates of growth of chicks. The gains of chicks in the various experiments were plotted against the concentrations of dispensable amino acids in the plasma of these chicks (fig. 1). Since the basal diets used in these studies contained only the indispensable amino acids, one would expect that chick growth would be limited by the level of dispensable amino acids supplied in the diet or by the amount that could be synthesized by the chick with a given diet. The correlation between growth rate and the concentration of dispensable amino acids of the plasma supports this expectation.

SUMMARY

Studies were conducted on the relative effectiveness of different sources of nitrogen for the synthesis of dispensable amino acids by the chick. A purified-type of diet containing only the indispensable amino acids was used in these studies. Growth, feed efficiency, nitrogen retention and plasma amino acid concentrations were the criteria used to determine the extent to which the different sources were utilized. Chicks fed amino acid diets that permitted gains in weight of 4 to 5 gm/day utilized urea and diammonium citrate to achieve growth which was as rapid, although possibly not as efficient as that achieved by the inclusion of dispensable amino acids in the diet. Feeding urea and ammonium citrate increased nitrogen retention and increased plasma levels of dispensable amino acids, indicating that the nitrogen of urea and ammonium citrate was retained and converted to dispensable amino acid nitrogen. When the levels of indispensable amino acids of the basal diet were increased so that more rapid growth was possible, urea was not as effective as the intact dispensable amino acids.

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Partial Reversal of beta-Aminopropionitrile Toxicity by Calcium in the Chick[1]

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Beta-aminopropionitrile produces striking anatomical lesions of the skeletal and vascular systems (Ponseti, '54). The chemical and anatomical effects of this compound, first isolated from the sweet pea by Shilling and Strong ('54), have been reported by many investigators. Levene and Gross ('59) showed that it increases fragility and solubility of collagen in the developing chick embryo.

The relationships of nutrients to β-aminopropionitrile toxicity have also been investigated by several workers. Dasler ('54) and Lee et al. ('56) reported that casein and gelatin minimize the lathyrogenic effects of β-aminopropionitrile on weanling rats. Zein was found to be ineffective. In another report (Dasler, '56) cysteine and glutamine were found effective in delaying the onset of toxicity, whereas a variety of other amino acids and carbohydrate derivatives were not effective. Liver powder, pyridoxine, calcium pantothenate and ascorbic acid were not found to alter the course of β-aminopropionitrile toxicity. Barnett and Morgan ('59) reported that high dietary fat levels increased the incidence of internal hemorrhage in growing chicks due to β-aminopropionitrile. Cholesterol feeding had no effect on the toxicity of the lathyrogenic agent. A preliminary report by Barnett and Morgan ('58) indicated that the hemorrhages produced by the toxic compound in chicks fed a high-fat diet could be increased by feeding additional pyridoxine and reduced by administration of deoxypyridoxine. Thornton ('60) found that dietary ascorbic acid increased the toxicity of β-aminopropionitrile for the turkey poult. Barnett et al. ('58) reported that vitamin K did not consistently reduce internal hemorrhage when added to a vitamin K-deficient diet containing toxic levels of the lathyrogenic agent. Amino acid supplements were found to be ineffective in altering abnormalities in chicks fed β-aminopropionitrile (Roy and Bird, '59). Working with crude feedstuffs, Waibel and Pomeroy ('59) found that fish meal hastened the incidence of hemorrhage in turkey poults from the toxic compound, β-aminopropionitrile toxicity, whereas tallow, grease, vegetable oils, wheat middlings, wheat bran, oats, alfalfa meal and dried whey did not hasten onset of hemorrhage.

The experiments reported herein were undertaken to study the toxic effect of β-aminopropionitrile on the developing chick embryo and to test a wide variety of compounds for potential effects in reversing the toxicity of the lathrogen in chick embryos and young growing chicks. Previous work by Chang et al. ('55) had shown that β-aminopropionitrile would produce bone deformities and lethal effects on chick embryos when administered on the fourth and tenth days of incubation.

EXPERIMENTAL

Fertile eggs from Leghorn-type breeding hens maintained with a complete diet were injected with β-aminopropionitrile fumarate[2] (BAPN) alone and in combination with other compounds prior to and during incubation. Procedures followed were identical to those described by Cravens and Snell ('49) except that solutions for injection were made from nonsterile compounds in previously sterilized water and equipment. All solutions were injected immediately after preparation at a volume of 0.1 ml/egg. Ten to 20 eggs were used for each treatment.

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Coenzyme A assays on chick embryo liver were conducted using the transacetylase method described by Stadtman et al. ('51). The enzyme was prepared from lypholized cells of *Clostridium kluyveri* as outlined by Stadtman and Barker ('49).

In other experiments day-old chicks were fed the basal corn-soybean meal-type ration shown in table 1 with and without BAPN and other supplements added at the expense of the corn. The birds were maintained in starting batteries and data on body weights, walking disability and mortality were obtained. In two of the trials, chicks were fed the basal ration; and water solutions of BAPN and calcium salts, alone and combined, were injected subcutaneously into the loose connective tissue between the femur and body wall, each chick receiving 0.2 ml daily for 7 days starting with day-old chicks.

TABLE 1

Composition of basal chick ration

	gm/kg
Ground yellow corn	651.3
Soybean meal (44% protein)	300.0
Sucrose	20.0
Defluorinated rock phosphate (31% calcium and 18% phosphorus)	15.0
Calcium carbonate	2.0
Iodized salt (0.007% iodine)	5.0
Manganese sulfate (27% manganese)	0.2
DL-Methionine	1.5
Premix[1]	5.0

[1] Premix supplied the following quantities/kg: vitamin A, 2200 USP units; vitamin D_3, 600 ICU; riboflavin, 4.2 mg; Ca-D-pantothenate, 8.4 mg; niacin, 20 mg; choline chloride, 500 mg; cyanobalamin, 12 µg; procaine penicillin, 4 mg; and butylated hydroxytoluene, 125 mg.

Difficulty in walking shown by birds treated with BAPN was measured by scoring the degree of disability as follows: 0 = normal; 1 = limp; 2 = severe limp, able to stand; 3 = crawl on hocks only, unable to stand; 4 = unable to move about the cage; and 5 = dead. This scoring procedure was found to be reliable and repeatable since each score was based upon clear-cut differences. The score was used to measure the reversal effect of certain compounds on BAPN's crippling action.

RESULTS

Data presented in table 2 show that mortality of chick embryos was closely related to the dose of BAPN administered. At levels of 1.0 mg/egg or more, BAPN administration resulted in 100% mortality prior to the sixth day of development. At levels of 0.4, 0.6 and 0.8 mg/egg, some of the embryos survived beyond the fifth day but none survived the entire incubation period. BAPN administered at a level of 0.2 mg/egg allowed about one-half of the embryos to hatch, whereas a level of 0.1 mg/egg permitted normal hatchability.

TABLE 2

Effect of β-aminopropionitrile dosages on mortality and hatchability of the developing chick embryo

Treatment[1] (dose/egg)	Embryo mortality		Hatch
	0–5 Days	6–20 Days	
	%	%	%
None	10	10	80
Water, 0.1 ml	10	20	70
BAPN,[2] 10 mg	100	0	0
BAPN, 1.0 mg	100	0	0
BAPN, 0.8 mg	95	5	0
BAPN, 0.6 mg	50	50	0
BAPN, 0.4 mg	35	65	0
BAPN, 0.2 mg	20	40	40
BAPN, 0.1 mg	10	10	80
BAPN, 0.01 mg	10	20	70
BAPN, 0.001 mg	15	20	65

[1] Treatment administered prior to incubation of eggs (20 eggs/treatment).
[2] β-Aminopropionitrile fumarate.

When large doses of BAPN were administered prior to incubation, its toxicity was characterized by the presence of hemorrhages both in the embryo proper and in the extra embryonic circulatory system. Hemorrhages were not confined to any specific location.

With lower levels (0.3 and 0.4 mg of BAPN) visceral and skin hemorrhages were not found in embryos injected at zero, 2 or 4 days of incubation. However, embryos injected at 8 and 15 days displayed both types of hemorrhages. Skin hemorrhages were concentrated in the feet and toes and around the eyes. Of the visceral hemorrhages bleeding into the pericardial sac was most common. Embryos injected at 15 days also had hepatic and general visceral hemorrhage. Embryos injected with the very large dose of 20 mg at 13 and 14 days developed massive hepatic hemorrhages within three hours after treatment. None of the embryos or chicks hatched from BAPN-in-

jected eggs showed aortic ruptures like those found in young chicks and turkey poults fed BAPN. When BAPN was given after 4 days of development, skeletal abnormalities typical of lathyrogenic agents were observed. Embryos killed by a massive dose of BAPN (10 to 12 mg/egg) were extremely fragile and the tissues were easily friable. Gross et al. ('60) have reported extensively on the fragility of tissues from BAPN-treated embryos and changes in collagen solubility of tissues resulting from BAPN administration.

Table 3 shows the calculated lethal dose required to produce 50% mortality (LD_{50}) in chick embryos when they are given graded doses of BAPN at different times during the incubation period. The amount of BAPN required for the LD_{50} increases with age when the entire egg is used as a reference except that the embryo appears to be most sensitive to BAPN when treated on the second day of development. If the LD_{50} is based on the wet weight of the embryo instead of the entire egg, the dose required to produce the LD_{50} decreases greatly during the first week of development and remains constant thereafter. Thus the amount of BAPN necessary to give the LD_{50} is closely related to the weight of the embryo during the second week of incubation but dependent on other factors during the initial week of development.

The results of a study to classify chick embryos dying from BAPN administration into precise developmental stages at the time of death is shown in table 4. BAPN was never found to alter or inhibit primary differentiation. Within the dosage range used, BAPN injected prior to incubation or after two days of incubation seldom stopped development prior to the third day. The data show that the embryos died during two periods, 3.5 to 5 days and 8 to 10 days, when treated at zero and two days of incubation. The major gross defect detected in those embryos ceasing development between stages 19 and 26 were alterations in the torsion and flexion, whereas those dying at stages 34 to 36 had twisted and shortened lower beaks. At stages 38 to 40 the embryos showed malformed legs typical of those associated with lathyritic agents.

In a series of experiments attempts were made to reverse the toxic effects of BAPN with a variety of vitamins, amino acids and other substances, some of which had previously been reported effective. Riboflavin, pyridoxine, deoxypyridoxine, folacin, choline, cyanocobalamin, menadione·$NaHSO_3$ complex, α-tocopheryl acetate, L-arginine, L-cystine, L-glutamine, a casein hydrolysate and versene did not alter the toxicity of 0.2, 0.3 or 0.4 mg of BAPN when simultaneously injected prior to incubation. Calcium (D-form) pantothenate at levels of 10 to 20 mg/egg was found to influence BAPN toxicity. Table 5 shows that this compound injected simultaneously with 0.4 mg of BAPN allowed 13 to 23% hatchability. In addition this treatment lengthened the sur-

TABLE 3

Median lethal dose (LD_{50}) of BAPN[1] in developing chick embryo[2]

Day of incubation at treatment	Approx embryo wt	LD_{50} in mg BAPN/egg				LD_{50} in mg BAPN/gm embryo	
		Day of incubation at which mortality was determined					
		5	10	18	22	18	22
	gm						
0	0.0002	0.37	0.27	0.23	0.13	1150.0	650.0
2	0.003	0.12	0.12	0.12	0.09	42.0	30.0
4	0.05		0.24	0.16	0.07	3.2	1.4
6	0.29		0.32	0.26	0.12	0.9	0.4
8	1.15			0.65		0.6	
10	2.26			1.34	0.48	0.6	0.2
15	12.00			7.50		0.6	

[1] β-Aminopropionitrile fumarate.

[2] LD_{50} values were determined from dose response curves which were obtained by injecting several doses of BAPN into the incubating egg at various periods of incubation. The LD_{50}'s at 5, 10, 18 and 22 days are from the cumulative per cent dead at the respective days of incubation.

TABLE 4

Developmental inhibitory effects of BAPN [1] *in the chick embryo*

Treatment dose of BAPN/egg	No. of fertile eggs	Day of incubation at treatment	Cumulative % of embryos dead at indicated stage of development																		
			Days of incubation at death																		
			3–3.5			3.5–4.5			4.5–5.5			5.5–7			7–8		8–9	10	11	12	13	14
			Stage of development at death [2]																		
			19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
mg																								
0.1	20	0														5								10
0.2	20	0							5	10	15					20	25	30		35		40		
0.3	20	0				5	10	25	30	40								55		65			70	
0.4	20	0				10	40	65	70	75		80				85			90		95		100	
0.5	20	0			10	25	60	75	85	90	95							100						
0.1	18	2				11	17		33															
0.2	17	2				12	29	59	76									82	88					
0.3	18	2			6	17	61	83									90		96					
0.5	19	2			16	26	58	74	84	100														

[1] β-Aminopropionitrile fumarate.
[2] Stages according to Hamburger and Hamilton ('51).

vival time of embryos dying prior to hatching over that observed for embryos injected with only the 0.4 mg level of BAPN. The reversal of BAPN toxicity by calcium pantothenate was by no means complete. In the experiments reported, however, calcium pantothenate had either increased survival time of embryos or allowed some chicks to hatch when they were given 0.4 mg of BAPN. A biochemical relationship of BAPN to pantothenic acid in unknown.

Experiments were next conducted to study compounds related to pantothenic acid for their effect on BAPN toxicity. Results of such experiments showed that β-alanine, D-pantethine and coenzyme A did not reverse or otherwise affect BAPN toxicity.

If BAPN produces its toxic effect through interference in pantothenic acid metabolism, coenzyme A levels in the embryo might be depressed by BAPN administration. Coenzyme A assays of liver from normal and BAPN-treated embryos were conducted. While the units of coenzyme A per milligram of liver were variable, there was no indication that massive doses of BAPN reduced liver coenzyme A concentration.

An early experiment had suggested that calcium in the form of calcium chloride did not reverse BAPN toxicity when used in amounts equimolar to those supplied by calcium pantothenate. A more extensive investigation of calcium compounds, however, revealed that calcium had an effect on the course of BAPN toxicity when the compounds were administered prior to incubation. Three experiments to test the effect of several levels of 4 calcium salts on mortality of embryos injected with and without BAPN are summarized in figure 1. Reduction in mortality of embryos from BAPN toxicity due to increased level of calcium administration was found to be very highly statistically significant ($P < 0.001$). Calcium salts administered in the absence of BAPN did not significantly affect mortality at 5 days of incubation.

Because several calcium compounds partially reversed BAPN toxicity in the embryo, feeding and injection experiments with young chicks were undertaken to de-

TABLE 5

Effect of calcium pantothenate on β-aminopropionitrile (BAPN) toxicity in the developing chick embryo[1]

Compound injected[2]		Embryo mortality		
BAPN	Calcium pantothenate	0–10 Day	11–20 Day	Hatch
mg/egg	*mg/egg*	%	%	%
0		8	4	88
(Water only, 0.1 ml)		8	11	81
0.4	0	84	16	0
0	10	25	12	63
0	20	32	12	56
0.4	10	50	27	23
0.4	20	62	25	13

[1] Pooled data from 4 experiments using 20 eggs/treatment in each experiment.
[2] Compounds administered prior to incubation of eggs.

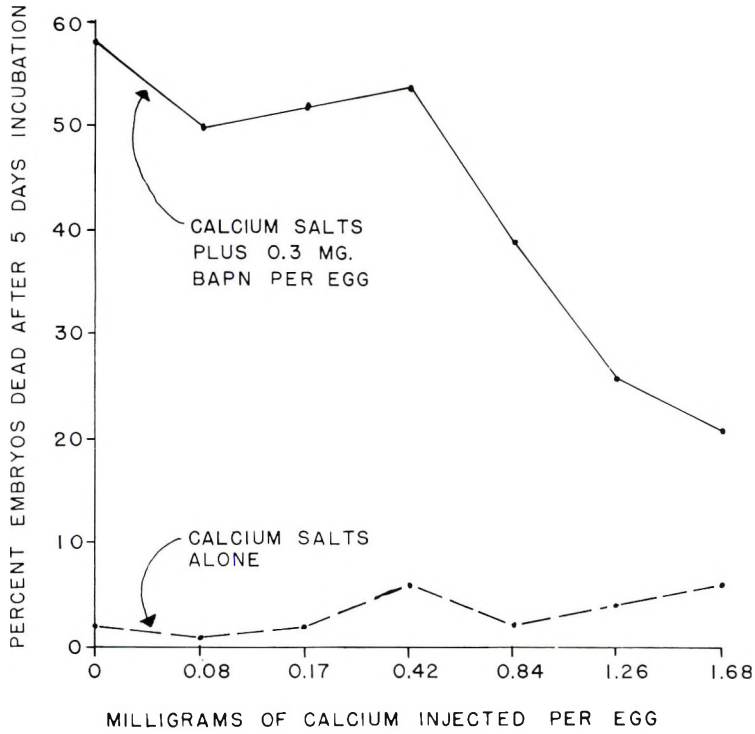

Fig. 1 Effect of calcium level on the toxicity of β-aminopropionitrile (BAPN) for the chick embryo when administered prior to incubation.

termine whether leg deformities and walking disability produced by BAPN could be influenced by calcium and other compounds. When 20 mg of BAPN were given to chicks by daily subcutaneous injection for the first 7 days of their lives, severe walking disability was induced. The data shown in table 6 reveal that calcium pantothenate and calcium gluconate significantly reversed the crippling effect of BAPN. Other experiments of this type showed that a combination of calcium pantothenate and calcium gluconate also is effective.

When growing chicks were fed 0.06% of BAPN in the diet, leg deformities and hemorrhagic disorders developed over a period of several weeks. Using this level

of the toxic compound, chicks were fed the basal ration shown in table 1 with and without a number of calcium sources and levels. In the first experiment the calcium content of the basal ration was adjusted to 0.92%. The data, shown in table 7, indicate that the higher calcium levels reduced walking disability and mortality of chicks fed BAPN. Growth was impaired by the high calcium levels when chicks were fed diets without BAPN. In the presence of BAPN, growth rate was poor but unaffected by the calcium level of the diet. The toxic manifestations of BAPN appeared to be influenced more by calcium level than calcium source. Therefore, a second experiment (table 8) was conducted with chicks fed 0.6 to 2.2% of calcium from the same source, with and without BAPN. The results of this experiment show clearly that walking disability and mortality due to BAPN is reduced as the calcium level of the experimental ration increases. As in the previous trial, increasing the calcium level reduced growth in the absence of BAPN but did not alter growth in the presence of the toxic agent.

In the chick trials 65 birds died prior to termination of the experimental period. Autopsies on these birds showed that death was due to aortic rupture or other hemorrhage in 27 chicks, and that most of the remaining chicks died from dehydration and starvation directly attributable to severe leg disability caused by BAPN.

TABLE 6

Influence of calcium compounds on BAPN-induced[1] walking disability in chicks

Exp.	No. of chicks	Daily treatment by injection	Disability score after indicated no. of treatments				
			3	4	5	6	7
1	10	20 mg BAPN	1.4	1.9	2.4	2.5	2.2
	10	20 mg BAPN + 20 mg Ca pantothenate	0.1	0.2	1.4	1.0	1.3[2]
	10	20 mg BAPN + 14 mg Ca gluconate	0.8	1.6	1.7	1.7	2.0[2]
2	20	20 mg BAPN	0.8	1.1	1.8	2.5	2.2
	20	20 mg BAPN + 20 mg Ca pantothenate	0.1	0.6	1.7	1.9	1.9[2]
	20	20 mg BAPN + 14 mg Ca gluconate	0.3	0.6	1.1	1.3	1.1[2]

[1] BAPN indicates β-aminopropionitrile fumarate.
[2] Reduction in walking disability due to calcium administration statistically significant ($P < 0.05$).

TABLE 7

Effect of dietary calcium source and level on growth, walking disability and mortality of growing chicks fed with and without 0.06% of BAPN[1,2]

Calcium salt used as supplement	Total calcium in diet	Avg body wt at 14 days of age		Disability score at 14 days of age		Mortality at 14 days of age	
		Without BAPN	With BAPN	Without BAPN	With BAPN	Without BAPN	With BAPN
	%	*gm*	*gm*			%	%
None	0.92	198	127	0	3.1	5	25
$CaCO_3 + CaHPO_4$	1.25	187	121	0	3.3	5	30
Ca pantothenate + $CaHPO_4$	1.35	192	124	0	3.5	0	35
Ca gluconate + $CaHPO_4$	1.80	173	128	0	2.0	0	15
Ca gluconate + $CaCO_3$ + $CaHPO_4$	1.86	157	112	0.1	1.7	0	10
$CaCO_3 + CaHPO_4$	1.92	159	131	0	2.1	5	5

[1] BAPN indicates β-aminopropionitrile fumarate.
[2] Twenty chicks/treatment.

TABLE 8

Effect of dietary calcium level on growth, walking disability and mortality in growing chicks fed with and without 0.06% of BAPN[1,2]

Calcium level in experimental ration (from $CaCO_3$)	Avg body wt at 20 days of age		Disability score				Mortality at 20 days of age	
			At 10 days		At 20 days			
	Without BAPN	With BAPN	Without BAPN	With BAPN	Without BAPN	With BAPN	Without BAPN	With BAPN
%	gm	gm					%	%
0.6	303	165	0.3	2.6	0	4.4	0	47
1.0	301	173	0	1.7	0	3.9	0	40
1.4	229	164	0.3	1.8	0.6	3.6	0	23
1.8	199	162	0.3	1.1	0.3	3.6	3	20
2.2	187	159	0.9	1.0	0.3	3.2	3	0

[1] BAPN indicates β-aminopropionitrile fumarate.
[2] Thirty chicks/treatment.

DISCUSSION

The observation that BAPN does not alter the pattern of primary differentiation of the chick embryo suggests that this compound does not act as an inhibtor of protein synthesis because cell division and tissue growth occur rapidly during this phase of early development. Toxicity of BAPN as reflected by embryonic death takes place only after the formation of an extensive vascular system. Disruptions in the vascular system appear to be the primary cause of death. Thus, only after formation of structures supported by a connective tissue framework does BAPN produce its lethal effect. The increased solubility of collagen from connective tissue due to BAPN has been adequately demonstrated by Levene and Gross ('59).

Partial reversal of BAPN toxicity by injected or dietary calcium is the primary observation resulting from this study. This reversal of BAPN toxicity is based upon increased survival time and a decrease in deformities produced by the lathyrogenic agent when calcium salts are administered. Although calcium can reverse the lethal effect of BAPN when injected as a single dose or fed for a limited period of time, its effect is much less apparent when the toxic agent is injected or fed continuously over an extended period of time. Thus, clear-cut effects of calcium can be demonstrated early in a continuous feeding or injection experiment but are difficult to show if the experiment is continued for three or more weeks.

The manner in which calcium produces its effect on BAPN toxicity is not clear. Because calcium is effective by injection or feeding and does not reduce growth in the presence of BAPN, it is probable that the reversal effect is concerned with the mineral metabolism of the animal. Dasler et al. ('61) have suggested that lathyrogens may change the physical nature of connective tissue by forming chelates. Levene ('61) has shown that not all lathyrogens are chelating agents. Additonal work on the effect of BAPN on calcium metabolism is needed.

Finally, it is possible that the increased incidence of internal hemorrhage caused by BAPN using high-fat diets as reported by Barnett and Morgan ('59), is due to the well known effect of dietary fat on reducing calcium availability. Thus the fat-fed birds probably had less calcium available than the birds fed diets without fat.

SUMMARY

Experiments with the developing chick embryo and growing chick were undertaken to study the toxic effect of β-aminopropionitrile fumarate (BAPN) and agents that might be capable of preventing or modifying the toxic condition produced by this lathyrogenic agent.

BAPN failed to disturb primary differentiation of the chick embryo and induced death only after this developmental phase had passed. The median lethal dose (LD_{50}) of BAPN decreased as embryo weight increased throughout the first

week of incubation, but remained constant during the second week of incubation.

Calcium compounds were found to partially reverse the incidence of walking disability and mortality due to BAPN injection or feeding. Other compounds tested for their effect on BAPN toxicity with negative results were: riboflavin, pyridoxine, deoxypyridoxine, folacin, choline, cyanocobalamin, menadione·$NaSO_3$ complex, α-tocopheryl acetate, L-arginine·HCl, L-cystine, L-glutamine, a casein hydrolysate, β-alanine, coenzyme A and versene.

The effect of calcium on BAPN toxicity was closely related to injected dose or dietary level of calcium but not to the calcium source. It is possible that BAPN interferes with the mineral metabolism of the bird and that calcium partially corrects this metabolic derangement.

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Effect of Pyridoxine Deficiency on Iron Absorption in the Rat[1]

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It has been reported previously that pyridoxine deficiency results in an increased iron absorption in swine (Cartwright et al., '44) and rats (Gubler et al., '49). This study was undertaken to investigate further the relationship between pyridoxine deficiency and iron uptake at various levels of iron intake.

MATERIALS AND METHODS

Weanling female Sprague-Dawley rats were fed a control or a pyridoxine-deficient diet. The latter consisted of vitamin-free casein, 18%; cottonseed oil, 5%; sucrose, 73%; and an iron-free salt mixture (Gubler et al., '49), 4%. The following vitamins were added at the levels indicated /100 gm of basal diet: vitamins A, D, and E at 400, 40 and 0.7 IU, respectively; thiamine, 0.5 mg; riboflavin, 0.8 mg; niacin, 4.0 mg; calcium pantothenate, 4.0 mg; biotin, 0.04 mg; folic acid, 0.20 mg; menadione, 0.50 mg; and inositol, 100.0 mg. The control diet was prepared by adding 0.5 mg of pyridoxine/100 gm of the basal diet.

The total iron uptake was determined by assay of the total body activity after ingestion of $Fe^{55\text{-}59}Cl_3$. The amount of iron in the administered radio-iron solutions was determined on the day of administration by the method of Elvehjem ('30). After sacrifice and removal of the stomach and intestines, the carcass was placed in 150 ml of a 25% (W/V) KOH-ethanol solution. This procedure solubilized all but the bones in about 8 hours. The bones were brought into solution separately in 5 N HNO_3.

The radioactivities of the digestion mixtures were determined by counting 50-ml aliquots in a Nuclear Chicago Model DS5-5 Scintillation well counter, using a Nuclear Chicago Model 132 analyzer-computer. The percentage recovery of the administered dose using this technique was determined in one experiment by assaying the activity in the bones, carcass, feces, and urine. In this experiment with 12 animals, a recovery of 97.2 ± 5% of the administered dose was obtained.

RESULTS

In the first experiment (table 1) the control and experimental animals were maintained with their respective diets for 5 weeks. Then 1 ml of a solution containing 0.1 mg of $Fe^{55\text{-}59}$ was administered by stomach tube to each animal. The rats were killed 24 hours later (ether) and the iron absorption was determined as described previously. The radioactivity of the blood was determined by withdrawing one milliliter from the heart at the time of sacrifice, diluting to 50 ml with one N HNO_3 and counting. On the basis of previous food intake the group designated pyridoxine-deficient plus pyridoxine supplement ingested 45 to 55 μg of pyridoxine /day for 4 days prior to the administration of the radio-iron.

A statistical analysis of the data indicates there is a significant difference ($P = < 0.05$) in the mean iron absorption of both the controls and the pyridoxine-supplemented animals when compared with the pyridoxine-deficient animals. These results suggest there is a decreased iron absorption in pyridoxine deficiency at the level of iron administered and for the period allowed for absorption of the radio-iron.

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TABLE 1

Amount of iron absorbed from a single dose (stomach tube) of 0.1 mg of radio-iron[1,2]

Group	Mean weight	Mean iron absorption	Mean activity of blood
	gm	*μg/100 gm body weight*	*count/min/ml*
Control	142 ± 5[3]	17 ± 3	140 ± 48
Pyridoxine-deficient	120 ± 16	9 ± 1	79 ± 20
Pyridoxine-deficient + pyridoxine supplement	141 ± 3	15 ± 3	98 ± 25

[1] 0.1 mg of the radio-iron had an activity of 108,900 count/min when assayed as described in Materials and Methods section of text.
[2] Three animals/group.
[3] Standard error of the mean.

TABLE 2

Amount of iron absorbed per day after ad libitum feeding of diets containing 1 mg of radio-iron[1]*/100 gm of diet for 14 days*[2]

Group	Mean weight	Mean daily iron absorption	Mean activity of blood
	gm	*μg/100 gm body weight*[3]	*count/min/ml*
Control	209 ± 16[4]	13.2 ± 1.0	2784 ± 321
Pyridoxine-deficient	156 ± 2	13.3 ± 1.8	2504 ± 467
Pyridoxine-deficient + pyridoxine supplement	188 ± 6	21.0 ± 2.0	3624 ± 94

[1] 0.1 mg of the radio-iron, assayed 24 hours after sacrifice, had an activity of 143,684 count/min.
[2] Four animals/group.
[3] Calculated on the basis of the body weight of each animal at the time of sacrifice.
[4] Standard error of the mean.

In the second experiment, after the animals had been fed their respective diets for 10 weeks, Fe^{55-59} was added to each diet at a level of 1 mg/100 gm of diet. This level of iron was intended to furnish each rat a daily intake of about 0.1 mg of radio-iron. Dietary pyridoxine supplementation to assure a level of intake of 45 to 55 μg/day of one-half of the deficient animals was begun on the day the iron was added to the diet. The animals were allowed to eat ad libitum during the 14-day experimental period. The total radio-iron ingested by each of the control and pyridoxine-supplemented animals was 1.5 mg, and the pyridoxine-deficient animals ingested 1.1 mg. At the end of the experimental period the rats were killed and the amount of iron absorbed was determined. The radioactivity of the blood was determined as described previously.

The results shown in table 2 indicate no difference in absorption of iron between the control and pyridoxine-deficient group. The increased iron intake by the group fed the pyridoxine supplement group can be attributed, at least in part, to increased hemoglobin synthesis as a result of the growth spurt induced by the addition of pyridoxine to the diet.

In the last experiment the animals had been fed their appropriate diets for 12 weeks, at which time sufficient salt mixture (Hubbell et al., '37) and radio-iron were added to bring the iron content of the diets to 10 mg of iron/100 gm of diet. Dietary pyridoxine supplementation to furnish an intake of 45 to 55 μg/day of one-half the group fed the deficient diet was begun at the same time, and during the remainder of the experiment (18 days) the animals were pair-fed to the pyridoxine-deficient group. The total iron ingested per animal was 14.5 mg.

Examination of the data in table 3 indicates there was a greater iron absorption by the animals fed the pyridoxine-deficient diet than by the controls ($P = < 0.05$). The pyridoxine - supplemented rats absorbed significantly more iron than did either of the other groups ($P = < 0.01$). This group also deposited significantly

TABLE 3

Iron absorption resulting from 18 days of feeding diets containing 10 mg of iron[1]/100 gm of diet (all animals pair-fed to the pyridoxine-deficient group)[2]

Group	Mean weight	Mean daily iron absorption	Mean iron content (bones)
	gm	*μg/100 gm body weight*[3]	*μg/100 gm body weight*
Control	190 ± 27[4]	61.5 ± 8.8	138 ± 22
Pyridoxine-deficient	158 ± 5	70.3 ± 7.6	144 ± 12
Pyridoxine-deficient + pyridoxine supplement	161 ± 6	82.0 ± 6.3	204 ± 43

[1] 0.1 mg of the radio-iron in the diet, assayed 24 hours after sacrifice, had an activity of 93,642 count/min.
[2] Four animals/group.
[3] Calculated on the basis of the body weight of each animal at the time of sacrifice.
[4] Standard error of the mean.

TABLE 4

Mean hemoglobin levels for the various groups in the three experiments

Exp. no.	Control	Pyridoxine-deficient group	Pyridoxine-deficient + pyridoxine supplement group
	gm/100 ml	*gm/100 ml*	*gm/100 ml*
1	15.2 ± 1.3[1]	15.0 ± 0.9	15.1 ± 1.1
2	13.7 ± 1.9	13.1 ± 1.2	13.3 ± 1.4
3	13.8 ± 1.7	13.0 ± 1.4	13.2 ± 1.5

[1] Standard error of the mean.

more iron in their bone marrow than did the control or deficient groups ($P = < 0.01$).

Hemoglobins (table 4) were determined as oxyhemoglobin in all three experiments using a Bausch and Lomb Spectronic 20 spectrophotometer. There were no significant differences in the hemoglobin levels of the various groups in any of the three experiments.

Urinary iron excretion was determined in experiments 1 and 2 and found to be negligible. Intestinal excretion of iron could not be determined inasmuch as dietary radio-iron was present in the gut throughout the entire period of each experiment. The values given for mean daily iron absorption are therefore subject to the error of the amount of iron re-excreted via the bile. It is probable, however, that this error is quite small.

DISCUSSION

These data indicate that rats fed pyridoxine-deficient diets do not absorb increased amounts of iron when their dietary iron intake is about 100 μg/day. If the iron intake is increased to 1.0 mg/day, pyridoxine-deficient animals absorb somewhat larger amounts of iron than do their controls. In the latter instance, our data agree with those of Gubler et al. ('49). These workers maintained their animals with the high-iron intake for a longer period of time, which may account for the relatively greater uptake of iron by their pyridoxine-deficient group in comparison with the controls. The decreased absorption of iron by the pyridoxine-deficient group seen in experiment 1 was confirmed by two additional experiments conducted under similar experimental conditions. At present we are unable to give an adequate explanation for the decreased absorption of iron during the first 24 hours after administration of iron to the animals fed the pyridoxine-deficient diet.

The question may be raised as to whether an iron intake of 1.0 mg/day by the rat is physiological. By comparison with the human it appears not to be so. Such an intake for a 200-gm rat is equivalent to a 350 mg/day intake for a 70-kg human. On the basis of human needs, it would seem reasonable to estimate that the iron requirement of the rat lies in the range of 50 to 100 μg/day. Accordingly, we consider on the basis of our experiments that enhanced absorption of iron in the pyridoxine-deficient rat does not occur under physiological conditions. It is of interest that most salt mixtures used in rat diets furnish daily intakes of 2 to 2.5 mg/day — intakes which are undoubtedly far in excess of actual needs.

We have found in preliminary studies that simultaneous administration of pyridoxine and iron to pyridoxine-deficient, iron-depleted animals results in a greatly enhanced absorption. This was also demonstrated in nondeficient, iron-depleted animals, but the enhancement was not as great. Furthermore, *in vivo* and *in vitro* studies are to be initiated to investigate the possibility of a direct role for pyridoxine in iron absorption.

SUMMARY

The absorption of Fe^{55-59} by rats fed pyridoxine-deficient, iron-free diets was studied under various conditions. The results indicated that pyridoxine deficiency did not enhance the absorption of iron when a single dose of 0.1 mg of iron was given by stomach tube or under conditions of iron intake considered to be physiological (50 to 100 μg/day). Conversely, at daily iron intakes of about 1 mg, the pyridoxine-deficient animals showed a significantly greater iron absorption. Under the last two experimental conditions mentioned above, pyridoxine supplementation of the deficient animals resulted in an iron absorption that was significantly greater than that for both the control and deficient animals.

ADDENDUM

During the preparation of this manuscript, Yeh et al.[2] reported that iron absorption is not affected by pyridoxine deficiency regardless of the severity of the deficiency or the dosage of iron given. The difference between their results and ours may be a result of the level of iron administered and the duration of the experiment.

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Effects of Asparagine and Other Related Nutritional Supplements upon Alcohol-Induced Rat Liver Triglyceride Elevation

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Administration of a single gastric dose of ethanol to rats has been reported from several laboratories to result in significant increases in liver triglyceride content (Mallov and Bloch, '56; Di Luzio, '58; Brodie et al., '61; Gillespie and Lucas, '61). Since L-glutamine had previously been found to prevent inhibition of growth of *Streptococcus fecalis* caused by ethanol (Ravel et al., '55) and to reduce the voluntary consumption of ethanol by rats (Rogers et al., '55), it was pertinent to study the effect of glutamine and related nutritional factors upon the rat liver lipid elevation induced by ethanol. In the present investigation,[2] reduction of the rat liver triglyceride elevation caused by a single administration of ethanol was used as a relatively rapid assay for possible protective activities of several simple nutritional supplements including glutamine, asparagine, and other related compounds. The results indicate that L-asparagine, administered together with the single gastric dose of ethanol, is effective in preventing subsequent liver triglyceride elevation, but glutamine and several other supplements are ineffective.

EXPERIMENTAL

Male white rats (Holtzman) in the 350- to 500-gm range were fed commercial laboratory chow[3] for 2 to 8 days between the time of receipt and experimental use. Six to 8 hours before treatment, food was removed from the cages, but water was available to the rats at all times. The test solutions were administered orally, from a hypodermic syringe through a rubber tube into the stomach of each animal. The concentrations of all components were adjusted so that the total volume given at each administration was 2.0 ml/100 gm rat. In some cases, as indicated, a supplementary solution was administered prior to the ethanol-containing dose, and in some cases dosage with a supplementary solution followed 4 to 5 hours after the ethanol dose.

After 16 to 18 hours, each rat was killed by a blow on the head, and the liver was removed, washed with water, blotted dry, and immediately frozen on solid carbon dioxide. After thawing, each liver was homogenized and its triglyceride content determined by the method of Butler et al. ('61), based on periodate oxidation of the glycerol moiety of the triglycerides.

RESULTS

The comparative effects of ethanol and glucose (in amount isocaloric with the ethanol) in increasing liver triglyceride content, and the comparative effects of L-asparagine and monosodium L-aspartate in counteracting the elevation, are shown by the data of table 1. Ethanol (4.8 gm/kg rat) induced a three- to fourfold increase in liver triglyceride content compared with that of the glucose-treated controls. Aspartate administered together with the ethanol had little effect upon this elevation, but a supplement of L-asparagine reduced the triglyceride level to 9.9 mg/gm of liver, an increase of approximately 16% compared with the elevation occurring after the dose of ethanol alone Neither asparagine nor aspartate when

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[2] A preliminary report has appeared: Lansford, E. M., Jr., I. D. Hill and W. Shive, Federation Proc., 21: 304 (1962).

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

administered together with glucose led to any significant alteration of the liver triglyceride level observed in animals given glucose alone.

In a separate experiment (experiment 3, table 2), a doubled amount of L-asparagine supplement, either in the form of a second dose of 0.2 gm of asparagine alone 4 hours following the initial dose of ethanol mixed with asparagine, or (in a different group of rats) in the form of a single dose of ethanol mixed with a supplement of 0.4 gm of L-asparagine/100 gm of rat, evidently afforded only slight additional protection against the liver triglyceride elevation, as compared with that of a single supplement of 0.2 gm of asparagine/100 gm rat. In parallel experiments, not tabulated, rat groups killed 12 hours after ethanol administration showed about the same triglyceride differential between ethanol-treated controls and asparagine-supplemented animals as those killed at 18 hours. Groups killed 24 hours after ethanol dosage showed little effect of asparagine; the differential appears to diminish as the liver lipid content falls back to the normal value. Groups that re-

TABLE 1

Effects of asparagine and aspartate upon ethanol-induced liver triglyceride levels

Dose	Supplement	Number of rats	Liver triglyceride content[1]
			mg/gm liver
Ethanol[2]	none	15	23.5 ± 5.3
Ethanol	L-asparagine[3]	15	9.9 ± 2.1
Ethanol	L-aspartate[4]	8	26.2 ± 7.3
Glucose[5]	none	15	7.4 ± 1.3
Glucose	L-asparagine	15	6.7 ± 2.1
Glucose	L-aspartate	10	8.7 ± 1.9

[1] Rats were killed 16 hours after dose was administered. Triglyceride values are tabulated as the mean of individual rat values plus or minus the standard error. For the comparison glucose vs. ethanol, $t = 11.1$, $P = 0.001$; for ethanol vs. ethanol-asparagine, $t = 8.96$, $P = 0.001$.
[2] Ethanol: 0.48 gm/100 gm rat, as a 31.5% v/v solution of 95% ethanol in water.
[3] Asparagine: 0.2 gm/100 gm rat, included in the ethanol or glucose solution. Most of the asparagine remained as a suspended solid phase during administration.
[4] Aspartate: 0.2 gm/100 gm rat; L-aspartic acid neutralized with NaOH to the monosodium salt, then included in the solution with ethanol or glucose.
[5] Glucose: 2.0 ml of a 48% solution/100 gm rat.

TABLE 2

Effects of various supplements upon ethanol-induced liver triglyceride elevation

Exp. no.	Dose[1]	Supplement	No. of rats	Hours after dose	Liver triglyceride
					mg/gm liver
3	Ethanol	none	8	18	22.2 ± 3.3
	Ethanol	L-asparagine	8	18	11.6 ± 5.3
	Ethanol	L-asparagine + second dose after 4 hours[2]	8	18	11.1 ± 4.0
	Ethanol	L-asparagine (double amount)[3]	8	18	9.8 ± 2.7
	Glucose	none	3	18	6.7 ± 1.8
4	Ethanol	none	8	18	22.8 ± 8.1
	Ethanol	L-aspartate (monosodium)	8	17	23.5 ± 5.0
	Ethanol	L-glutamate (monosodium)	8	18	20.9 ± 6.3
	Ethanol	L-glutamine	8	18	21.9 ± 8.4
	Ethanol	glycine	8	17	21.9 ± 3.2
	Ethanol	sodium citrate	8	18	28.7 ± 5.9
	Ethanol	α-ketoglutarate (monosodium)	8	17	22.6 ± 4.0
	Glucose	none	3	18	7.1 ± 0.9

[1] Ethanol dose in each case, 0.48 gm ethanol in 2.0 ml solution/100 gm rat.
[2] Initial dose included 0.48 gm ethanol and 0.2 gm L-asparagine/100 gm rat; second dose after 4 hours contained only L-asparagine, 0.2 gm/100 gm rat.
[3] Single dose, contained 0.48 gm ethanol and 0.4 gm L-asparagine/100 gm rat. All other supplements, 0.2 gm/100 gm rat, included in the ethanol solution; the supplement in some cases remained as a partly undissolved, suspended solid phase.

ceived an oral dose (0.2 gm/100 gm of rat) of asparagine alone 2.5 hours prior to a dose of ethanol alone, or asparagine alone 4 hours after a dose of ethanol alone, showed little effect of the asparagine on liver triglyceride elevation.

A number of other amino acid supplements tested have no significant effect upon liver triglyceride elevation; these include aspartate, glutamate, glutamine, and glycine (experiment 4, table 2). Further data, not tabulated, showed that a supplement of cystine is similarly inactive, and a supplement of methionine is only slightly active, in preventing liver lipid elevation. Supplements of a citric acid cycle intermediate were in two cases (citrate and ketoglutarate, table 2) ineffective; in another experiment, a supplement of sodium pyruvate was ineffective. However, in the latter experiment (not included in table 2) a supplement of α-ketoglutarate (monosodium salt; 0.2 gm/100 gm of rat) was partially effective in preventing the triglyceride elevation.

Other than the experiment including methionine just mentioned, the classic lipotropic agents were not studied in this investigation, since the initial objective was the possibility of further elucidation of the nature of the protective effect of glutamine (or perhaps closely related compounds) against certain toxic effects of alcohol, and furthermore, since Di Luzio and Zilversmit ('60) reported that ethanol-induced increase in neutral fat in rat liver was not prevented by choline chloride, either oral or intravenous.

DISCUSSION

These data demonstrating a prevention by L-asparagine of the liver triglyceride elevation induced by ethanol are at present stage evidently quite empirical, and the underlying metabolic mechanism involved can only be the subject of speculation. One explanation that appears possible is that asparagine provides to the animal a 4-carbon intermediate that accelerates ethanol metabolism by increasing the capacity of the citric acid cycle to oxidize 2-carbon units derived from ethanol, in the animals receiving the supplement. If this is true, it would be necessary to assume that asparagine is more readily assimilable, or less rapidly destroyed, than some of the other amino acid supplements that proved ineffective. Possibly a slow rate of absorption of asparagine in the digestive tract would be a factor in prolonging the maintenance of appropriate concentrations. Favoring the explanation involving the citric acid cycle is the partial effectiveness in one experimental group (although not in the experiment in table 2) of an intermediate of the cycle, α-ketoglutarate, as a supplement to the ethanol dose. This concept of increase in citric acid cycle oxidizing capacity as a basis for more effective alcohol removal is not apparently compatible with the generally accepted consideration (Westerfeld, '61) that alcohol dehydrogenase activity is the rate-limiting step in alcohol oxidation in higher animals. Although alcohol-fed rats were reported (Dajani and Orten, '62) to show an increased liver capacity for oxidation of ethanol in liver incubates, associated with increased concentrations of all the citric acid cycle intermediates, these responses were observed after a period of several weeks, and may not necessarily be related to the much more rapid responses involved in acute ethanol intoxication.

Other possible explanations of these observed effects of asparagine supplementation would need to take into account the considerable evidence (Mallov and Bloch, '56; Brodie et al., '61) that the induction of liver triglyceride elevation by ethanol is a complex, indirect physiological chain of interactions involving in turn pituitary and adrenal stimulation, depot tissue lipid mobilization, free fatty acid circulation in the blood, and a rate of liver glyceride accumulation exceeding the rate of liver glyceride loss. The data of the present study do not distinguish between these steps as possible sites of asparagine activity in preventing the final observed effect of ethanol, inasmuch as experiments have not yet been carried out to determine whether asparagine may influence the previously known action of adrenocorticotropic hormone in elevating liver lipid content, for example, nor to determine whether asparagine may affect the observed mobilization of depot tissue lipid components caused by adrenal products. Since the first of the hormonal responses in

rats following high doses of ethanol is a relatively rapid one, as shown by data indicating an increase in plasma corticosterone reaching maximal value by one-half hour after administration of ethanol (Brodie et al., '61), evidently a rather rapid action of asparagine in effecting ethanol removal would necessarily be involved in any postulation of a direct effect of asparagine upon ethanol blood levels in the supplemented animals. Alternative possibilities include asparagine action antagonistic to ethanol at the site of its initiation of the hormonal response chain, or inhibition by asparagine of any of the subsequent steps in this physiological chain of responses that in the absence of the supplement would lead to liver triglyceride accumulation.

SUMMARY

Male white rats which had received a single oral dose of ethanol (4.8 gm/kg of rat) showed, 16 to 18 hours later, liver triglyceride levels elevated three- to four-fold over those of control animals receiving isocaloric glucose; this triglyceride elevation was almost completely prevented by the inclusion of a supplement of L-asparagine (2 gm/kg of rat) in the ethanol solution administered. Asparagine administered separately (either 2.5 hours previously or 4 hours subsequently) from the ethanol had little effect in preventing liver triglyceride elevation; similarly ineffective was the administration, in the ethanol solution, of supplements of any of a number of other amino acids (aspartate, glutamate, glutamine, glycine, cystine, methionine) or intermediates closely related to the citric acid cycle (citrate, ketoglutarate, pyruvate). Neither asparagine nor aspartate had any effect upon liver triglyceride content of control animals receiving glucose only.

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Studies on the Requirements of Young Poults for Available Phosphorus

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The reports of the Committee on Animal Nutrition of the National Research Council, dealing with the Nutrient Requirements for Poultry (revised, '54 and '60) indicate the phosphorus requirement of starting poults to 8 weeks of age to be one per cent of total phosphorus, with further stipulations as follows: "At least 0.5% of the total feed of starting poults should be inorganic phosphorus. All of the phosphorus of non-plant feed ingredients is considered to be inorganic. Approximately 30% of the phosphorus of plant products is non-phytin phosphorus and may be considered as part of the inorganic phosphorus requirement."

Singsen et al. ('47) concluded from their studies with poults that the phosphorus of a mixture of corn, wheat middlings and wheat bran is relatively unavailable for bone calcification, and found, furthermore, that the percentage of bone ash obtained in turkey poults was a straight line function of the logarithm of the non-cereal phosphorus when the diet contained from 0.2 to 0.67% of non-cereal phosphorus. Motzok and Slinger ('48) presented evidence indicating that young turkey poults fed high-energy diets required a minimum of 0.7 to 0.9% of dietary phosphorus. Later, Slinger and associates ('52, '55) reported that both vitamin E and phosphorus helped to reduce the incidence of perosis in young poults, with best prevention being achieved only when the diets contained 0.7% or more of *inorganic* phosphorus and 5 IU of supplementary vitamin E/454 gm of feed. Gillis and associates ('57) showed in studies with radioactive phosphorus that the phosphorus in calcium phytate is almost completely unavailable to the young turkey poult. Wilcox and associates ('54, '55) presented evidence of wide variability in the availability to young poults of the phosphorus in many *inorganic* feed sources of phosphorus.

Thus, it has become evident that in calculating the available phosphorus in diets for young poults it is necessary to consider the poor availability of the phosphorus in organic materials and also the variation in its availability in the different inorganic sources present in the starting diet. This concept came forcibly to our attention in studies with young poults fed purified diets. We found very poor utilization by poults of the phosphorus in reagent grade anhydrous dicalcium phosphate ($CaHPO_4$). Since the solubility characteristics of this material are very similar to those of commercial dicalcium phosphate, which shows a high phosphorus availability, it was considered desirable to determine the reason for the poor biological availability of the phosphorus in pure anhydrous dicalcium phosphate ($CaHPO_4$).

Several possibilities were investigated, as follows: (1) the phosphorus in anhydrous dicalcium phosphate ($CaHPO_4$) may exist in a chemical or physical form that resists solubilization, assimilation, or conversion to a utilizable form; (2) crude forms of phosphate and other natural feedstuffs may contain an unknown factor or trace mineral, not present in reagent grade anhydrous dicalcium phosphate, which is necessary for maximal utilization of phosphorus; (3) for maximal utilization of dicalcium phosphate, certain amounts of highly available phosphorus such as that present in the ash of natural materials, or supplied by the soluble phosphates such as potassium or sodium phosphates, may

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[2] On leave from the College of Agriculture, University of the Philippines, Los Banos, Philippines.

be needed. The studies presented in this report, therefore, were conducted (1) to obtain further information on the requirements of young poults for available phosphorus; (2) to assess the availability of the phosphorus in various materials; and (3) to determine whether interactions exist between various phosphates and other factors, which affect the total availability of phosphorus in diets of young poults.

EXPERIMENTAL

Empire Broad Breasted White poults of mixed sex from the Cornell turkey breeding flock were used in all experiments. The poults were housed in thermostatically controlled battery brooders with wire-mesh floors. Each brooding pen contained a 15 watt electric light shining directly into the feeding pan to assure maximal feed consumption. Feed and water were supplied ad libitum. One series of experiments was conducted with a purified diet. For the second series, a semi-purified diet was formulated by replacing the isolated soybean protein used in the purified diet with low-fiber, defatted commercial soybean oil meal containing 50% of protein. The composition of the basal diets is shown in table 1. The purified diet containing isolated soybean protein contained approximately 0.28% of phosphorus. When commercial soybean meal was substituted for isolated soybean protein, the resulting diet was found to contain 0.55% of phosphorus. Bone ash determinations were made at 550°C on the moisture-free, fat-free tibiae which were carefully cleaned so that the cartilage cap was retained.

RESULTS AND DISCUSSION

Studies with poults using the purified diet containing anhydrous reagent grade

TABLE 1
Basal diets

	Diet no. 1	Diet no. 2
	%	%
Cornstarch	38.89	13.24
Isolated soybean protein[1]	41.00	—
Dehulled soybean meal, 50% protein	—	69.70
DL-Methionine	0.65	0.65
Glycine	0.50	0.50
Cellulose[2]	3.00	—
Fat (partially hydrogenated vegetable fats)	7.00	7.00
Mineral mixture	3.91	3.91
Vitamin mixture	1.00	1.00
DPPD	0.044	0.044
Total	96.00[3]	96.00[3]

Vitamins	*mg/kg diet*
Niacin	88
Ca pantothenate	22
Riboflavin	10
Pyridoxine	10
Thiamine·HCl	10
Folic acid	2.2
Biotin	0.22
Choline chloride	2475
Vitamin E (*d*-α-tocopheryl acetate)	66
Vitamin K (menadione sodium bisulfite)	20
	units/kg diet
Vitamin A (stabilized)	13,200 IU
Vitamin D_3	4,400 ICU

Minerals	*% in diet*
$CaCO_3$	2.00
KCl	0.70
NaCl	0.90
$MnSO_4 \cdot H_2O$	0.05
$MgSO_4$	0.244
$FeSO_4 \cdot 5H_2O$	0.002
$ZnCl_2$	0.010
$CoCl_2 \cdot 6H_2O$	0.0004
Total	3.9064

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.
[2] Solka Floc, Brown Company, Berlin, New Hampshire.
[3] Calcium and/or phosphate supplements plus corn starch were added to make up the final 4% of the diets. Phosphorus content of basal purified diet no. 1 was found to be 0.28%. Diet no. 2 contained 0.55% of phosphorus.

dicalcium phosphate. Following an initial observation that when the purified diet contained reagent grade dicalcium phosphate at levels high enough to meet the National Research Council recommended allowances for both total and inorganic phosphorus, the poults showed symptoms of rickets at about three weeks of age, an experiment was undertaken in which the basal purified diet was fed alone and supplemented with graded levels of reagent grade anhydrous dicalcium phosphate in comparison to a known adequate level (0.75%) of commercial dicalcium phosphate.

The results of this experiment, presented in table 2, show that the phosphorus in the commercial dicalcium phosphate was considerably more available than that in the reagent grade anhydrous dicalcium phosphate. This confirmed the earlier observations that the level of 0.75% of added phosphorus, which was completely adequate when the highly available commercial phosphate was used, did not prevent rickets in the poults when reagent grade anhydrous dicalcium phosphate was used as the sole source of added phosphorus.

To make certain that the rickets that occurred in the poults receiving the anhydrous dicalcium phosphate was not due to a deficiency of vitamin D_3, an experiment was conducted using 0.9% of this source of phosphorus and supplementing the diet with various sources and amounts of vitamin D_3. The results of this experiment, presented in table 3, demonstrate that the failure of the reagent grade anhydrous dicalcium phosphate to prevent rickets was not due to an insufficient amount of vitamin D_3 in the diet.

The poults receiving the anhydrous phosphate at supposedly adequate levels showed symptoms similar to the enlarged hock

TABLE 2

Comparison of availability of phosphorus in reagent grade anhydrous dicalcium phosphate and commercial dicalcium phosphate

Treatments	Avg poult wts, 3 weeks	Ash of fat-free dry tibia
	gm	*%*
Basal diet no.1 (containing 0.28% P) + reagent grade anhydrous dicalcium phosphate to supply:		
0.45% P	169	24.9
0.60% P	235	27.2
0.75% P	247	39.4
0.90% P	286	40.7
Basal diet no. 1 + commercial dicalcium phosphate[1] to supply:		
0.75% P	280	47.8

[1] Dynafos, manufactured by International Minerals and Chemical Corporation, Skokie, Illinois.

TABLE 3

Failure of high levels of vitamin D_3 to promote normal calcification in poults receiving diet 1 plus 0.9% of phosphorus from reagent grade anhydrous phosphate

Experiment no.	Source of vitamin D_3	Amount of vitamin D_3	Bone ash
		ICU/kg diet	*%*
2	Irradiated animal sterols A[1]	2200	33
2	Irradiated animal sterols A	2200	36
5	Irradiated animal sterols A	4400	40
4	Irradiated animal sterols B[2]	4400	35
5	Irradiated animal sterols B	4400	38
5	Irradiated animal sterols B	44,000	39
5	Cod liver oil[3]	800	33
5	Cod liver oil	4400	36

[1] Delsterol, manufactured by E. I. duPont de Nemours and Company, Wilmington, Delaware.
[2] Nopdex, manufactured by Nopco Chemical Company, Harrison, New Jersey.
[3] Manufactured by Squibb and Company, New Brunswick, New Jersey.

disorder which occurs in commercial flocks of turkeys, and which has been studied extensively in this laboratory (Scott, '50, '51, '53). It was considered possible that this disorder also was the same as that studied by Hunt et al. ('54) who found that poults receiving a purified diet containing isolated soybean protein[3] showed a perosis-like condition which could be prevented by washing the isolated soybean protein or by replacing it on a protein basis with commercial soybean meal.

Accordingly, a series of experiments was conducted in which the isolated soybean protein used in the experimental purified diet was replaced in part or wholly with various other proteins such as washed isolated soybean protein, crude commercial soybean meal, casein, dried brewer's yeast, or with sources of unidentified chick growth factors (UGF).

The ashes of these materials also were fed in order to determine the effects of their total phosphorus when converted to the inorganic form. In addition, studies were made of the possibility that selenium, zinc, bromine, inositol, *p*-aminobenzoic acid, and antibiotic or some other trace mineral might be required for optimal utilization of the phosphorus in pure anhydrous dicalcium phosphate.

The results of these experiments, presented in table 4, showed that addition of selenium, zinc, bromine, extra trace minerals, vitamins and antibiotics, or washing of the isolated soybean protein, had no effect upon calcification. However, substitution of casein, dried brewer's yeast, commercial soybean meal or UGF sources for part or all of the isolated soybean protein caused marked improvements in calcification of the tibiae in the young poults. Since the ashes of the UGF and of the soybean meal also effectively improved calcification, this indicated that the mineral content of some portion of the UGF mixture and the soybean meal was largely responsible for the improvement produced. This effect did not appear to be present in grass juice or its ashes. Soybean meal at the 20% level, 10% of dried brewer's yeast and the 4 UGF sources each provided approximately 0.15% of phosphorus, as determined by chemical assay. While the phosphorus in dried brewer's yeast is known to be quite readily available, that in soybean meal and corn distiller's dried solubles (the major phosphorus source in the UGF mixture) is generally considered to be relatively unavailable. It is difficult, therefore, to account for the fact that the intact soybean meal at the 20% level and the intact UGF supplement produced calcification equal to that obtained when the ashes of these materials were added at equivalent levels; that this calcification was superior to that obtained with dried brewer's yeast or its ash, and better when the diet contained 0.9% of phosphorus rather than 0.6% from the reagent grade anhydrous dicalcium phosphate. It appears from these results that either the phosphorus in soybean meal is very highly available or that the meal contains some unknown property capable of improving the utilization of the phosphorus in the basal diet.

To confirm further the effect upon calcification of intact soybean meal and to determine whether any non-phosphorus nutrients in soybean meal affect the utilization by poults of reagent grade anhydrous dicalcium phosphate, a series of experiments was conducted in which graded amounts of soybean meal were substituted in diet 1, on a protein basis, for isolated soybean protein and cornstarch. Other lots of poults received diet 1 plus amino acids, phospholipids, or dipotassium phosphate equivalent to the amounts of these substances which might be present in 20% of soybean meal or its ash.

The results of these studies, presented in table 5, showed that the amino acids and phospholipids had no effect upon calcification, but that 0.15% of phosphorus from dipotassium phosphate produced the same improvement in calcification as was obtained with an equal amount of phosphorus from the ash equivalent to 20% of soybean meal in the diet, and that the 20% of intact soybean meal increased calcification at least as well as its ash.

It was not possible from the data obtained here to determine whether the addition of KH_2PO_4 enhanced the availability of the phosphorus in the reagent grade anhydrous dicalcium phosphate. Edwards

[3] Drackett 220, now produced by Archer-Daniels-Midland Company, Minneapolis.

TABLE 4

Tibia ash of poults receiving diet 1 containing reagent grade anhydrous dicalcium phosphate and various supplements

Treatments[1]	Levels of added phosphorus in diet, %				
	0.45	0.6	0.75	0.9	1.05
	% ash of fat-free, dry tibia				
Basal diet no. 1	25(2)[2]	27(4)	35(2)	37(11)	41(1)
Double amounts of trace minerals in diet no.1		30(1)			
Trace minerals equivalent to those present in commercial dicalcium phosphate		31(1)			
Selenium (0.1 ppm) as sodium selenite			33(1)		
Zinc (100 ppm) as $ZnCl_2$			37(1)		
NaBr (125–500 ppm)				35(3)	
Inositol (1 gm), *p*-aminobenzoic acid (20 mg), + aureomycin·HCl (100 mg)/kg of diet				36(1)	
Brewer's dried yeast, 10%				41(4)	
Ash equivalent to 10% of brewer's yeast				39(1)	
Casein (20%), substituted for 20% isolated soybean protein		37(1)		45(1)	
Substitution of casein (100%) for isolated soy protein				49(1)	
Dehulled, 50% protein soybean meal (20%), substituted on a protein basis		38(1)		46(3)	
Ash equivalent to 20% of soybean meal		42(1)		46(2)	
Substitution of soybean meal (100%) for isolated soybean protein (diet 2, table 1)		46(1)		50(2)	
Ash equivalent to100% of soybean meal substitution		47(1)			
4 UGF[3]				46(1)	
Ash equivalent to 4 UGF				45(1)	
Dried grass juice, 3%		29(1)			
Ash equivalent to 3% of dried grass juice		28(1)			

[1] Unless indicated otherwise, all supplements added at expense of cornstarch.
[2] Figures in parentheses show number of experiments from which average value is taken.
[3] Mixture containing 6% distiller's dried solubles, 3% dried grass juice, 3% dried whey product and 3% fish solubles.

et al. ('58) presented evidence in similar experiments indicating that the available phosphorus in these two materials is simply additive for promotion of growth and calcification in young poults. However, even though the results of the present experiments show that calcification was markedly improved by the presence of a small amount of highly available phosphorus from potassium acid phosphate, it is nevertheless difficult to account for the very beneficial effect upon calcification brought about by 20% of intact soybean meal.

Following a preliminary report of some of these studies (Scott et al., '56), Gillis ('57) and Gillis et al. ('62) reported on a series of experiments on the same subject. In studies with young poults, these investigators confirmed the poor availability of reagent grade anhydrous dicalcium phosphate, but showed that pure dicalcium phosphate *containing two molecules of water of hydration* exhibited excellent

TABLE 5

Effects of soybean meal, various nutrients in soybean meal and other substances upon calcification in poults receiving 0.9% phosphorus from reagent grade anhydrous dicalcium phosphate

Lot no.	Treatment	No. of experimental lots	Ash of fat-free dry tibia
			%
1	Basal diet no. 1 (0.9% P from $CaHPO_4$)	11	37(31–40)
2	Soybean meal, 5%	1	39
3	Soybean meal, 10%	1	41
4	Soybean meal, 20%	4	46(44–48)
5	Soybean meal, 69.7%	2	50(50–50)
6	Amino acids equivalent to 20% of soybean meal	2	39(38–40)
7	L-Glutamine, 0.625%	1	38
8	Cystine, 0.25%	1	36
9	Crude soy lecithin, 0.1%	1	36
10	Purified phosphatides from soy, 0.1%	1	39
11	Ash equivalent to 20% of soybean meal[1]	4	45(42–48)
12	Acid-soluble portion of soy ash equivalent to 20% of soybean meal[2]	2	44(43–45)
13	KH_2PO_4 to supply 0.15% P together with 0.75% P from $CaHPO_4$	1	49
14	Commercial dicalcium phosphate, 0.75%, substituted for 0.9% $CaHPO_4$	1	48

[1] Ash equivalent to 20% soybean meal supplied 0.15% phosphorus (probably as potassium phosphate). Thus for the treatments where the ash or its derivatives were added, the amount of reagent grade anhydrous dicalcium phosphate was reduced to supply only 0.75% phosphorus.

[2] Eight kilograms of soybean meal ashed to produce 100 gm of ash; major portion of ash was dissolved by boiling in 300 ml 1:3 HCl; cooled, filtered, evaporated to dryness on steam bath. Analysis of diet containing this filtrate equivalent to 20% soybean meal showed total phosphorus equal to that of lot 11.

availability of phosphorus. In view of this, further studies were conducted in our laboratory to determine the relative effectiveness of the phosphorus in anhydrous and hydrated dicalcium phosphates.

The results of the experiment, presented in table 6, show that hydrated USP dicalcium phosphate produced excellent calcification, while the reagent grade anhydrous dicalcium phosphate produced poor calcification when fed to provide equal amounts of phosphorus under similar environmental conditions.

TABLE 6

Anhydrous versus hydrated $CaHPO_4$ as sources of phosphorus for young poults

Treatments	Total phosphorus in diet	Ash of fat-free dry tibia
	%	%
Basal diet no.1 + $CaHPO_4$ to supply 0.75% P	1.03	37
Basal + $CaHPO_4 \cdot 2H_2O$ to supply 0.75% P (average of 5 lots)	1.03	48

These results indicate, therefore, that the chemical or physical structure of the anhydrous phosphate renders its phosphorus less available for young poults as compared to that in hydrated dicalcium phosphate.

Studies on availability to young poults of phosphorus in various inorganic phosphates. Having shown a marked difference in availability to young poults of the phosphorus in two different kinds of inorganic, chemically pure dicalcium phosphate, supposedly differing only in that one sample was hydrated while the other was not, it was considered desirable to test the availability of the phosphorus in a variety of inorganic phosphates in common use in commercial poultry feeding. Since it was desired to obtain results that could be applied to practice, diet 2 containing intact soybean meal as the source of protein was used for these studies. A series of experiments was undertaken using diet 2 alone and supplemented with various feed phosphates.

The results of the experiments presented in table 7 show that even with intact soy-

TABLE 7

Availability of phosphorus in various inorganic phosphates

Treatment	Supplemental phosphorus	Ash of fat-free dry tibia
	%	%
Basal diet no. 2 (containing 0.55% P, largely from soybean meal)	0	all dead
+ $CaHPO_4$	0.23	28(50)[1]
+ $CaHPO_4$	0.30	32(16)
+ $CaHPO_4$	0.46	39(9)
+ $CaHPO_4$	0.69	46(14)
+ $CaHPO_4$	0.92	47(16)
Commercial dicalcium phosphate	0.30	38(16)
Commercial dicalcium phosphate	0.45	47(12)
Commercial dicalcium phosphate	0.90	49(0)
Defluorinated rock phosphate	0.46	45(8)
Steamed bone meal	0.46	40(0)
Curaçao Island phosphate (avg 4 trials, using two samples)	0.46	38(14)
Colloidal (soft) phosphate	0.46	26(83)

[1] Figures in parentheses represent per cent mortality during the experiment.

bean meal as the protein source, there were wide differences in the availability of the phosphorus in the various inorganic feed phosphates.

DISCUSSION

Since 0.9% of reagent grade anhydrous dicalcium phosphate added to the isolated soybean protein diet failed to produce normal calcification (tables 2 and 4), whereas addition of 0.69% of this phosphate to diet 2 produced near-maximal results, it appears either (1) that the availability of the phosphorus in soybean meal is much higher than previously suspected, or (2) that soybean meal contains some unknown factor which greatly enhances the availability of the phosphorus in reagent grade anhydrous dicalcium phosphate, or (3) that the isolated soybean protein[4] contains a principle which inhibits utilization of this form of phosphorus.

Since addition of 0.15% of phosphorus from KH_2PO_4 produced maximal calcification when used with 0.7% of phosphorus from reagent grade anhydrous dicalcium phosphate, it appears that the presence in the diet of a small amount of highly available phosphorus may enhance the availability of the phosphorus in less well utilized sources — such as pure anhydrous dicalcium phosphate. However, attempts by Edwards et al. ('58) and by Nelson and Peeler ('61) to enhance the availability of certain low-availability phosphates by feeding them to poults and chicks in combination with potassium phosphate, ash of UGF or commercial dicalcium phosphate, failed to show any improvement in biological value of the low-availability phosphates.

The studies presented in this report do not lend themselves to the establishment of a more accurate requirement of young poults for available phosphorus. They show, however, that availability of the phosphorus in both organic and inorganic sources in the diet must be known before the diet can be properly assessed in terms of its ability to meet the requirement.

When very highly available phosphorus sources are used, the NRC ('60) recommended level of 1.0% of total phosphorus is adequate. However, when availability of phosphorus in *either* the *organic or inorganic* phosphorus sources in the diet is reduced, the requirement of young poults for total phosphorus may be considerably higher. Since studies by a number of laboratories have shown that excessive amounts of highly available phosphorus are inhibitory to growth and cause leg weaknessses, it is extremely important that supplements of known availability be used at correct levels in poultry diets, especially in those for young poults.

[4] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.

SUMMARY

Experiments on availability of phosphorus for young poults showed that the phosphorus in reagent grade anhydrous dicalcium phosphate ($CaHPO_4$) is relatively unavailable as compared with that in hydrated dicalcium phosphate ($CaHPO_4 \cdot 2H_2O$) or in commercial dicalcium phosphate.

The results indicated either (1) that the availability of the phosphorus in commercial soybean meal is much higher than previously suspected, or (2) that soybean meal contains some unknown factor which enhances the availability of the phosphorus in reagent grade anhydrous dicalcium phosphate.

Even in a diet containing intact soybean meal as the source of protein, there were marked differences in the availability to young poults of the phosphorus in various inorganic feed phosphates.

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Influence of Rat Strain and Protein Level on Protein Efficiency Ratio (PER) Determination

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During the year 1959, our laboratory participated in a collaborative study on the evaluation of protein quality sponsored by the Association of Official Agricultural Chemists (AOAC). The method chosen for this study (Derse, '58) was that of rat growth with protein efficiency ratio (PER) calculation. An important part of this method was the provision, suggested earlier by the Canadian Food and Drug Directorate (Chapman, et al., '59),[1] that the PER's obtained with test proteins should be multiplied by the fraction.

$$\frac{2.5}{\text{Determined PER of reference casein}}$$

The purpose of this provision was to reduce the variability among results obtained in various laboratories.

We were interested in establishing the validity of this procedure of correcting PER'S relative to casein, in particular when the quality of the test protein is limited by a different amino acid than is that of casein. Since it appeared reasonable that much of the variation in results among different laboratories was a result of strain differences in the rats, we decided to investigate this question by obtaining 8 different rat strains and feeding each one three different protein sources. We also investigated the influence of protein level on PER since in the past this seemed pertinent in comparing animal and cereal proteins. These factors were also recently investigated by Morrison and Campbell ('60).

EXPERIMENTAL

The rats used in these experiments were individually housed in screen-bottom cages in an air conditioned room maintained at approximately 75°F. Food and water were supplied ad libitum and the rats were weighed weekly over the 4-week experimental period. Food consumption was determined for each rat weekly, and a daily record of food spillage was kept. The rats, after receipt, were fed overnight with stock diet[2] and water ad libitum. They were then randomly assigned to groups which were adjusted to give similar average weights (for each strain). All rats were 22 or 23 days old at the start of the experiment and all were males.

In the first experiment rats from 8 strains[3] (designated A through H) were divided into groups (three groups/strain, 10 rats/group) and fed diets with the protein source, ANRC casein,[4] rolled oats or wheat gluten supplemented with 3.4% of LMH.[5] The diets were made up according to the proposed AOAC method (Derse, '58). The analyses of the protein sources for moisture, nitrogen, fat, lysine and threonine are shown in table 1.[6] The diets contained an average of $9.72 \pm 0.16\%$ of protein (10.14% dry weight). This is

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[1] Trade Information Letter, no. 156 1957 Department of National Research and Welfare, Ottawa, Canada.

[2] D and G Rat and Mouse Diet, Dietrich and Gambrill Inc., Frederick, Maryland.

[3] Rat strains used in these experiments have been designated as follows: (A) Charles River C-D, (B) Sprague Dawley, (C) Carworth Nelson, (D) Hemlock Hallow Wistar, (E) Canadian Food and Drug Directorate Wistar, (F) Hemlock Hallow Sherman, (G) Rockland Sherman, and (H) Rockland Long Evans.

[4] Animal Nutrition Research Council (ANRC) casein obtained from Sheffield Chemical Company, Norwich, New York.

[5] Du Pont lysine monohydrochloride containing 95% of the L-isomer. The supplementation level of amino acids in these experiments is calculated on the basis of the dry weight of the wheat gluten.

[6] Nitrogen (Kjeldahl) and fat (ether extract) were determined by Gascoyne and Company, Baltimore, Maryland. Lysine and threonine were determined by ion exchange chromatography (Moore and Stein, '51).

[7] For experiments 2 and 3 the vitamin mixture used was Vitamin Diet Fortification Mixture, obtained from Nutritional Biochemicals Corporation, Cleveland. In experiment 1, the vitamin mixture was made up as specified by Derse ('58).

TABLE 1

Analysis of protein sources

Protein source	H_2O	N	Protein[1]	Fat	Lysine	Threonine
	%	%	%	%	*gm/16 gm N*	*gm/16 gm N*
ANRC[2] casein	7.95	13.88	86.75	0.07	—[3]	—[3]
Rolled oats, exp. 1[4]	9.99	2.61	16.32	6.86	2.56	—[3]
Rolled oats, exp. 2[4]	9.41	2.72	17.00	6.81	2.93	1.94
Wheat gluten[5]	7.21	13.41	83.81	0.93	1.64	2.08

[1] Protein = N x 6.25.
[2] Animal Nutrition Research Council casein, obtained from Sheffield Chemical Company, Norwich, New York.
[3] Not determined.
[4] Quaker Heavy White no. 1 oats.
[5] Pro-80, General Mills, Inc., Minneapolis.

TABLE 2

Composition of basal diets

Experiment 1		Experiments 2 and 3	
	%		%
Sample	A[1]	Fat (corn oil to make)	10
Cottonseed oil	8 − (A × % ether extract)/100	Cellulose,non-nutritive[2]	5
Water	5 − (A × % moisture)/100	Salt, USP 14	4
Salts, USP 14	5 − (A × % ash)/100	Vitamin mixture[3]	2
Cellulose, non-nutritive	1 − (A × % crude fiber)/100	Cornstarch[5] q.s.	100
Vitamin mixture[4]	1		
Sucrose q.s.	100		

[1] A = 9.09 x 100/% protein in sample (N x 6.25).
[2] Alphacel, Nutritional Biochemicals Corporation.
[3] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.
[4] See Derse ('58).
[5] Sample added at expense of cornstarch.

approximately 0.1% more nitrogen than specified by Derse, primarily because of slight nitrogen contributions by dietary ingredients other than the protein source.

Experiments 2 and 3 were carried out with Charles River C–D rats (strain A). These diets were made up according to Chapman et al. ('59) except that a commercial vitamin mixture was used and the protein level of the diets was varied from 9 to 16% dry weight.[7] in experiment 2, the protein sources were ANRC casein, rolled oats and wheat gluten supplemented with 3.4% of LMH. For each protein source 7 groups of 9 rats were used. In experiment 3, the protein source, wheat gluten supplemented with 3.4% of LMH and 1.0% of L-threonine, was fed to 8 groups over the protein range mentioned above with 9 rats/group. The composition of the basal diets used in these experiments is shown in table 2.

RESULTS

Influence of rat strain. It was recognized that a major risk in bringing 8 rat strains together into one animal room was that of disease resulting from cross infections. Animals that died, failed to gain weight or appeared sick in any way were eliminated from the groups. All rats of strains A, B, and C survived the experimental period in good health; of the other strains, the number of rats surviving the experimental period in good health are as follows: strain E 29 out of 30; strains D and F 27 out of 30; strain G 25 out of 30; and strain H 24 out of 30. In experiments 2 and 3 mortality was zero.

The growth curves obtained in experiment 1 for the various strains therefore, are not to be considered as optimal performance for these particular strains. However, since the main purpose of this experiment was to determine the validity

TABLE 3

Weight gains for 8 strains of rats fed three protein sources (4 weeks' growth)

Rat strain	Parent strain[1]	ANRC Casein		Rolled oats		Wheat gluten + 3.4% LMH [2]	
		Initial wt	Wt gain	Initial wt	Wt gain	Initial wt	Wt gain
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
A	Sprague-Dawley	50.0	108.9 ± 20.9[3]	50.0	80.6 ± 9.6	50.0	50.6 ± 15.0
B	Sprague-Dawley	49.2	86.7 ± 14.2	49.1	59.2 ± 10.2	49.2	30.1 ± 5.9
C	Wistar	49.5	84.4 ± 16.7	49.5	68.8 ± 15.4	49.6	34.1 ± 10.8
D	Wistar	59.9	72.2 ± 23.2	59.3	55.8 ± 12.7	60.1	25.1 ± 11.0
E	Wistar	35.1	53.0 ± 7.7	34.9	33.7 ± 5.3	35.2	23.7 ± 3.8
F	Sherman	62.5	78.1 ± 19.8	62.2	64.0 ± 13.2	62.4	33.7 ± 10.9
G	Sherman	50.9	72.1 ± 14.5	50.9	50.6 ± 16.2	50.4	23.5 ± 9.8
H	Long-Evans	47.3	48.5 ± 5.3	47.9	35.3 ± 7.1	49.1	11.7 ± 6.8
Average weight gains			75.5 ± 19.2 (25.4%)[5]		56.0 ± 16.0 (28.6%)[5]		29.1 ± 11.3 (38.9%)[5]
Adjusted weight gains[4]					55.7 ± 4.8 (8.6%)[5]		28.4 ± 5.6 (19.7%)[5]

[1] These are the parent strains from which the actual strains used were derived. For the key to the strain designations A to H see footnote 3 in text.

[2] DuPont lysine monohydrochloride.

[3] Standard deviation.

[4] Weight gains for rolled oats and gluten + LMH adjusted relative to casein according to formula:

$$\text{Adjusted weight gain, sample}_{(\text{strain n})} = \text{weight gain, sample}_{(\text{strain n})} \times \frac{75.5}{\text{Weight gain, casein}_{(\text{strain n})}}.$$

[5] Coefficient of variation.

and efficacy of the procedure of correcting PER's obtained with a standard reference casein, it is not believed that the mortality encountered in experiment 1 interfered in this respect.

The average weight gains for the 24 groups in experiment 1 are shown in table 3. The standard deviations for these weight gains are quite high, both within each group and also among the 8 strains. The variability is in general the greatest for those groups showing the poorest growth. The differences in growth rates among the 8 strains are great but nevertheless are consistent for the three diets fed each strain. This is illustrated by the reduction in the coefficient of variation for rolled oats and gluten plus lysine from 28.6 and 38.9% to 8.6 and 19.7%, respectively, when the growth is considered relative to the growth attained by each strain on ANRC casein.

TABLE 4

Protein efficiency ratios[1] for 8 strains of rats

Strain	Protein efficiency ratios				Adjusted PER[2] 4 weeks
	1 Week	2 Weeks	3 Weeks	4 Weeks	
			ANRC casein		
A	3.51	3.67	3.22	3.24	—
B	3.60	3.30	2.85	2.97	—
C	3.48	3.04	2.88	3.04	—
D	1.70	2.45	2.47	2.64	—
E	2.49	2.79	2.72	2.68	—
F	2.27	2.59	2.64	2.65	—
G	2.54	2.68	2.64	2.77	—
H	2.31	2.19	2.03	2.20	—
Average	2.74	2.84	2.68	2.77	—
Standard deviation	0.70	0.48	0.34	0.32	—
Coefficient of variation	25.5%	16.9	12.7	11.6	
			Rolled oats		
A	2.75	2.64	2.66	2.63	2.25
B	2.17	2.47	2.31	2.25	2.10
C	2.18	2.24	2.38	2.49	2.27
D	1.79	2.04	2.00	2.13	2.23
E	1.61	1.83	1.83	1.89	1.96
F	2.03	2.22	2.14	2.06	2.15
G	1.56	1.97	2.15	2.08	2.08
H	1.15	1.40	1.68	1.80	2.26
Average	1.90	2.10	2.14	2.17	2.16
Standard deviation	0.49	0.39	0.31	0.28	0.11
Coefficient of variation	25.8%	18.6	14.5	12.9	5.1
			Wheat gluten + 3.4% lysine monohydrochloride (LMH)		
A	1.75	1.96	2.12	1.97	1.68
B	0.92	1.43	1.59	1.59	1.48
C	1.25	1.40	1.64	1.63	1.49
D	0.62	1.04	1.09	1.15	1.21
E	0.90	1.28	1.40	1.42	1.52
F	0.46	1.04	1.25	1.44	1.51
G	0.38	0.98	1.31	1.31	1.31
H	0.05	0.50	0.65	0.71	0.90
Average	0.79	1.20	1.38	1.40	1.39
Standard deviation	0.54	0.42	0.43	0.37	0.24
Coefficient of variation	68.3%	35.0	31.2	26.4	17.3

[1] Protein efficiency ratio (PER) = $\frac{\text{gm weight gained}}{\text{gm protein consumed}}$.

[2] PER's for rolled oats and gluten + LMH adjusted relative to casein accorded to formula:

$$\text{Adjusted PER, sample}_{(\text{strain n})} = \text{PER sample}_{(\text{strain n})} \times \frac{2.77}{\text{PER for casein}_{(\text{strain n})}}.$$

The protein efficiency ratios (PER's) corresponding to these weight gains are listed in table 4. The PER's are calculated for experimental periods of one through four weeks. The numerical value for the PER does not vary much over this period for casein and rolled oats, although it does for gluten plus 3.4% lysine. For all three diets there is a gradual lowering of the coefficient of variation as the experimental period is increased with a stabilization occurring after three to four weeks. The procedure of adjusting PER's relative to the PER obtained with casein, as for the weight gains, reduces the coefficient of variation from 12.9 to 5.1% for oats and from 26.4 to 17.3% for gluten plus lysine.

In table 5 the weight gains and PER's obtained with strain A are listed as cal-

TABLE 5

Weight gains and protein efficiency ratios (PER's) at 1 through 4 weeks, strain A

Protein source		1 Week	2 Weeks	3 Weeks	4 Weeks
ANRC casein	Wt gain:	21.3 ±4.5[1]	51.8 ±10.7	75.2 ±16.1	108.9 ±20.9
	PER:[2]	3.51±0.65[1]	3.67± 0.25	3.21± 0.25	3.24± 0.18
Rolled oats	Wt gain:	16.6 ±1.9	35.3 ± 6.39	58.2 ± 7.72	80.6 ± 9.59
	PER:	2.75±0.22	2.64± 0.23	2.65± 0.12	2.63± 0.13
Gluten + 3.4% LMH[3]	Wt gain:	10.0 ±5.0	23.6 ± 9.03	41.5 ±12.4	50.6 ±15.0
	PER:	1.74±0.68	1.96± 0.41	2.11± 0.32	1.97± 0.24

[1] Standard deviation.
[2] Grams weight gain/gram protein consumed.
[3] DuPont lysine monohydrochloride.

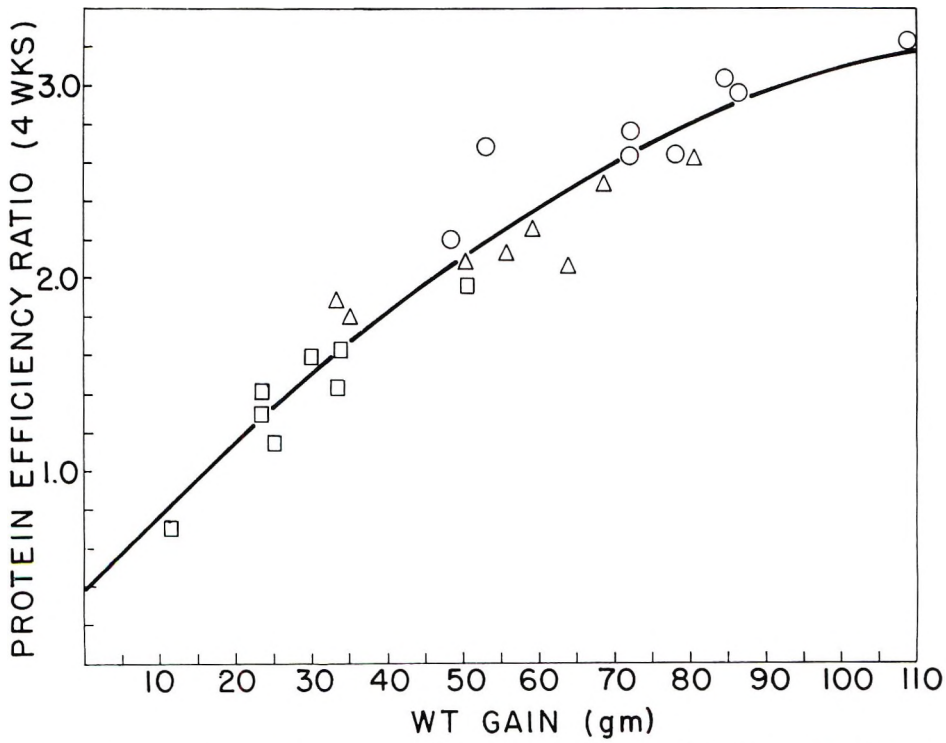

Fig.1 Relationship between protein efficiency ratio and 4-week weight gains, three protein sources, 8 rats strains. Key: ○ indicates ANRC casein; △, rolled oats; □ wheat gluten + 3.4% of lysine monohydrochloride (LMH).

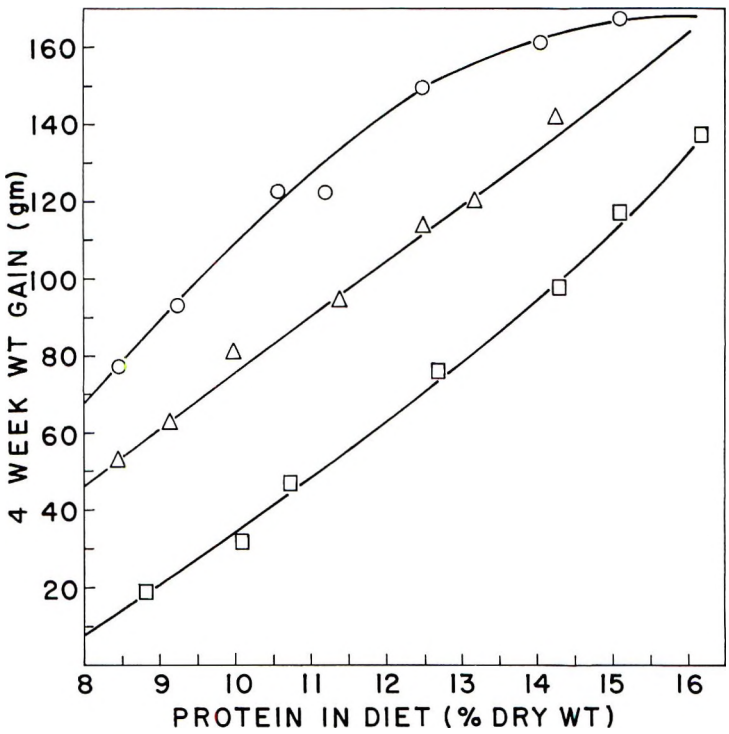

Fig. 2 Effect of dietary protein level on 4-week weight gains, strain A. Key: ○ indicates ANRC casein; △, rolled oats; □, wheat gluten + 3.4% lysine monohydrochloride (LMH).

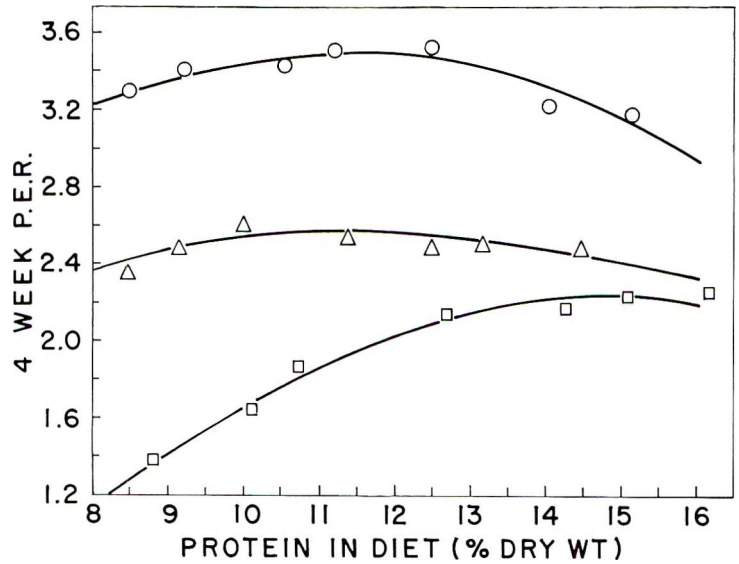

Fig. 3 Effect of dietary protein level on PER (4 weeks' growth), strain A. Key: ○ indicates ANRC casein; △, rolled oats; □, wheat gluten + 3.4% lysine monohydrochloride (LMH).

culated for one through four weeks' growth. The rat-to-rat variation in PER, similar to strain-to-strain variation, in all cases gradually decreases over the 4-week period.

In figure 1 the PER's obtained in experiment 1 with the 8 strains each fed three protein sources are plotted against the corresponding three weight gains. Considering the biological variation encountered in an experiment of this type, the correlation of PER as a function of weight gain, independent of strain or protein source, is striking. Using the method of least squares, the best quadratic expression that could be fitted to these data is $y = 0.390 + 0.041x - 0.000144x^2$. The correlation coefficient is $r = 0.966$.

Effect of protein level. In experiment 2, the same three protein sources were fed to male 22-day-old strain A rats with

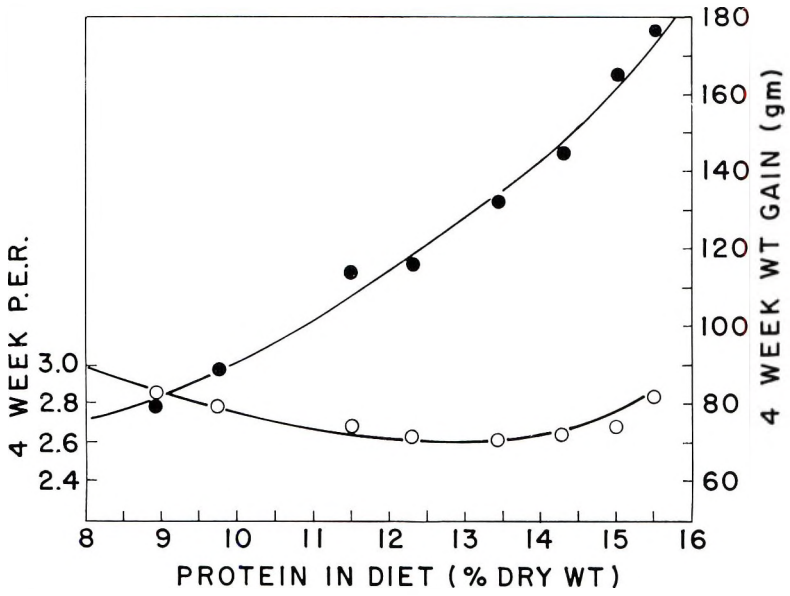

Fig. 4 Effect of dietary protein level on PER and weight gain (4 weeks' growth), wheat gluten supplemented with 3.4% LMH and 1.0% L-threonine, strain A. Key: ● indicates weight gain; ○, protein efficiency ratio (PER).

TABLE 6

Lysine and threonine content of cereal diets (10% protein, dry wt)

Diet	L-Lysine		L-Threonine		Lysine/ threonine	PER[1]
	% dry wt	*gm/16 gm N*	*% dry wt*	*gm/16 gm N*		
Wheat gluten	0.16	1.6	0.21	2.1	0.8	0.51
Wheat gluten + 3.4% LMH[2]	0.46	4.6	0.21	2.1	2.2	1.68
Wheat gluten + 3.4% LMH + 1.0% L-threonine	0.46	4.6	0.32	3.2	1.4	2.19
Rolled oats	0.29	2.9	0.19	1.9	1.5	2.25
Rat requirement, Rose et al. ('49)	1.0	8.0	0.5	4.0	2.0	—
Rat requirement, Rama Rao et al. ('59)	0.9	10.1	0.5	5.5	1.8	—

[1] Protein Efficiency Ratios obtained with strain A rats corrected relative to an assigned value for casein of 2.77.
[2] DuPont lysine monohydrochloride.

the protein level in the range 9 to 16% dry weight. The weight gains as plotted against protein level are shown in figure 2. The corresponding PER's are shown in figure 3. The PER's for casein and oats were found to be relatively little affected by protein level in the protein range measured. The PER obtained with wheat gluten supplemented with 3.4% of LMH, in contrast, is quite sensitive to protein level, particularly in the 8 to 13% range. It seemed probable that this response was caused by the fact that one amino acid, other than lysine, was limiting growth severely at low-protein levels, but at higher protein levels was being supplied in more nearly adequate amounts. It was determined experimentally that the limiting amino acid was threonine. Therefore in the third experiment wheat gluten supplemented with 3.4% of LMH and 1.0% of L-threonine was fed to 22-day-old male strain A rats for 4 weeks with the protein level varied as in experiment 2. Both the PER and weight gain curves are shown in figure 4. In this case the PER, as for casein and oats, is relatively independent of the dietary protein level, varying only 0.2 of a PER unit over the entire protein range measured.

The lysine and threonine content of the wheat gluten and rolled oats diets at 10% protein are listed in table 6 along with the corresponding PER's. The low PER obtained with wheat gluten supplemented with 3.4% of LMH is somewhat unexpected in view of the fact that the gluten diet furnished more threonine than the oats diet, according to chemical analysis. It is possible that some of the threonine in the wheat gluten[8] used as in the case of rice, is unavailable to the weanling rat. This phenomenon is not observed when white bread is supplemented with lysine (Rosenberg et al., '60). The rolled oats and wheat gluten plus LMH plus threonine diets at 10% protein both gave about the same corrected PER's and had about the same lysine/threonine ratio. However, the rolled oats diet contained only 60% as much lysine or threonine as the supplemented wheat gluten. Other factors besides availability of threonine may be involved in the lower response with gluten, but the data are too few to permit an answer and more work is required.

DISCUSSION

The results of the 1959 AOAC collaborative study on the evaluation of protein quality have been published (Derse, '60). The PER responses obtained with ANRC casein as reported by Derse for 11 collaborators, 2.79 ± 0.34 (range 2.15 to 3.31) agree remarkably well with our results obtained with 8 strains, 2.77 ± 0.32 (range 2.20 to 3.24). However, in contrast with the AOAC collaborative study, where no reduction in the coefficient of variation for test protein was obtained by correcting relative to casein, we found a very considerable reduction in variability resulted when the PER's obtained with the two test proteins were adjusted relative to casein. Our test proteins were limiting in threonine and lysine, respectively, while casein is limiting in sulfur amino acids. Our data show no distortion in PER is introduced by the correction procedure. Correcting the weight gains themselves relative to the weight gain on casein was equally efficacious in reducing strain-to-strain variability. The variation of PER with rat strain and its reduction by adjusting the PER's obtained with test proteins relative to the PER obtained with ANRC casein, was noted previously by Morrison and Campbell ('60).

The procedure of correcting the PER's obtained with test proteins relative to that obtained with a standardized casein was used some time ago by Friedman and Kline ('50). This correction procedure appears to be a useful means to eliminate variability in PER determination and should be a part of any standard PER method. The data in figure 1 show that regardless of rat strain or protein source, PER is a function of growth. This fact has been used by previous workers as an argument that PER calculation represents only added time and trouble and gives no advantage over using gain in body weight (Hegsted and Worcester, '47; Sherwood and Weldon, '53). This argument, however, neglects two main advantages PER calculation offers over gain in body weight. These are the lowered coefficient of varia-

[8] Pro-80, General Mills, Inc., Minneapolis.

tion for PER's as compared with weight gains, and the fact, which will be discussed below, that in the range of 9 to 16% of dietary protein, PER is less sensitive to changes in protein level than is weight gain. The lowered coefficient of variation for PER compared with weight gain also has been noted by Morrison and Campbell ('60).

It has been shown for casein, rolled oats and gluten supplemented with lysine and threonine that the PER is relatively insensitive to change in protein level in the range of 9 to 16% of dietary protein. The distinction that has often been made between animal and vegetable proteins appears to result only from the lower level of the limiting essential amino acids in the vegetable protein. The increase in PER as the level of wheat gluten plus lysine in the diet is raised appears clearly to result from the fact that as the protein level is raised, the limiting amino acid threonine is furnished in more nearly adequate amounts. Barnes et al. ('45), using rats, measured the change in PER over the protein range 5 to 40%. From their data they have concluded that "the common practice of employing a 10% protein diet, regardless of the nature of the protein, will result in a considerable distortion of nutritive values, and the magnitude of the error will increase as the nutritive quality of the protein decreases." Our data show a very pronounced dependence on protein level for the PER obtained with wheat gluten supplemented with 3.4% of LMH. Our data clearly show, however, that this results from a threonine limitation in this protein. Comparing maximum PER's, as originally suggested by Osborne et al. ('19) and again by Barnes and Bosshardt ('46), tends to obscure the differences in protein quality that can be shown up readily at lower protein levels. Dietary protein in the range 10 to 12% dry weight appears to us to be the most satisfactory level at which to assay protein quality by the PER method.

SUMMARY

The influence of two main variables, rat strain and protein level, in protein efficiency ratio (PER) determination using male weanling rats was studied. Strain-to-strain variation in PER (grams of weight gain per gram protein consumed) was very marked, with values ranging from 2.20 to 3.24 obtained for ANRC casein as calculated after 4 weeks' growth. The procedure of correcting PER's obtained with test proteins relative to that obtained with ANRC casein was found to greatly reduce strain-to-strain variability in PER determination.

The PER's obtained with ANRC casein, rolled oats, and wheat gluten supplemented with 3.4% of LMH and 1.0% of L-threonine were found to be relatively insensitive to changes in protein level in the range 9 to 16% dry weight. On the other hand, the PER obtained with wheat gluten supplemented with lysine alone was strongly dependent on protein level increasing from 1.4 at 9% of protein to 2.2 at 15% of protein (dry weight). This latter result was found to result from a threonine limitation which becomes less severe at higher protein levels.

ACKNOWLEDGMENTS

The author gratefully acknowledges the assistance of Vincent Bachetta who performed all the calculations in connection with the statistical analysis of these data. Also, the cooperation of Drs. J. A. Campbell and A. B. Morrison of the Canadian Food and Drug Directorate, Ottawa, Canada, who furnished us with a shipment of weanling rats from their own colony was greatly appreciated.

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Free Amino Acids in Dog Blood and Gut Contents After Feeding Meat[1]

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Earlier work (Nasset et al., '55) demonstrated that the qualitative free amino acid composition of gut contents is not greatly altered by the type of meal fed. The mixture present during digestion appears to contain amino acids derived from food proteins, autodigestion of hydrolytic enzymes, and other endogenous proteins (Nasset, '57). Ingesta are mixed with relatively large quantities of endogenous protein in the small intestine and therefore ingested protein does not greatly influence the molar ratios of free amino acids in gut contents (Nasset and Ju, '61).

The present work was designed to study the concentration of free amino acids in blood and gut contents sampled simultaneously after feeding meat.

EXPERIMENTAL

Six mongrel dogs were fasted 24 hours before taking blood from the saphenous vein and feeding. Blood was heparinized and centrifuged immediately after collection.

Experiment A. Saphenous blood (40 ml) was taken on days one and three from dogs one and two. Dog two was then fed 200 gm of ground lean beef and two hours later blood was again taken from the saphenous vein. The 40-ml samples in this series permitted duplicate determinations of free amino acids and related compounds.

Experiment B. Saphenous blood (20 ml) was taken from dogs 2 to 6 just prior to feeding them 200 gm of ground lean beef. They drank water at will. The dogs were anesthetized with pentobarbital sodium 1.5 hours after feeding. The abdomen was opened and clamps placed at cardia and pylorus. The portal vein and a mesenteric vein, approximately 50 cm caudad from the ligament of Treitz, were intubated. Two hours after feeding, blood was taken simultaneously from the carotid artery and jugular, portal, and mesenteric veins. The contents of that portion of the jejunum drained by the mesenteric vein were collected by washing out the lumen three times with distilled water. Contents and washings were combined, quickly frozen and lyophilized.

Each sample of plasma and gut contents was analyzed for free amino acids and related compounds by ion-exchange resin column chromatography with an automatic instrument similar to the one described by Spackman et al. ('58). Total N was determined by micro-Kjeldahl, NPN by nesslerization according to Gradwohl ('43), and Na and K by flame photometry.

RESULTS

Results of experiment A are given in table 1. Each value is the mean of duplicate analyses which differed by less than 5%. These preliminary experiments were conducted to observe what variations to expect in amino acid concentration of plasma from a peripheral vein of a fasting dog. Individual amino acids varied considerably in the same animal on different days and in different animals. All amino acids except phenylalanine, aspartic acid and glycine increased after the test meal in dog two. Although phenylalanine did not increase, tyrosine did.

Feeding produced two types of responses in experiment B. In three dogs (group 1) most of the free amino acids in plasma increased after the test meal. In the two other dogs (group 2), they decreased. The

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TABLE 1

Free amino acids and related compounds in dog saphenous vein plasma (exp. A)[1]

Amino acids and related compounds	Fasting				2-Hour postfeeding	
	Dog 1		Dog 2		Dog 2	
	S 1	S 2	S 1	S 2	S 1	S 2
Arginine	12.0	6.7	11.0	15.5	19.1	23.7
Histidine	8.9	7.8	8.8	10.7	15.7	19.1
Isoleucine	8.2	10.2	7.9	11.1	23.8	27.4
Leucine	11.7	11.9	9.6	12.3	26.1	31.0
Lysine	13.0	19.0	14.2	23.6	34.4	61.1
Methionine	3.6	3.7	3.3	2.6	6.9	8.2
Phenylalanine	6.6	3.4	8.6	19.2	7.0	16.2
Threonine	32.2	38.0	36.2	30.9	60.1	66.6
Tryptophan	2.3	trace	trace	trace	4.6	8.7
Valine	17.9	17.6	12.6	13.2	27.2	36.1
Alanine	41.0	52.5	46.6	34.8	64.2	85.0
Aspartic acid	2.1	1.1	0.0	0.0	0.0	0.0
Glutamic acid	1.5	1.4	3.3	3.1	3.9	3.9
Glycine	50.0	27.5	27.5	47.0	28.4	35.8
Proline	16.3	14.1	20.3	14.3	26.8	29.9
Serine	12.1	10.8	14.3	11.4	16.0	20.2
Tyrosine	4.0	3.3	5.1	7.0	14.6	21.4
Total free amino acids	243	229	229	257	379	494
Asparagine	98.8	126.5	113.0	113.0	115.0	150.0
Citrulline	0.0	3.3	2.9	3.5	4.6	2.5
Cysteic acid	3.0	1.9	1.6	2.1	2.1	4.8
Ethanolamine	0.0	0.0	0.0	0.0	3.7	1.6
Ornithine	2.0	2.0	2.5	3.2	6.2	7.6
Taurine	5.3	1.5	1.7	1.3	1.8	1.2

[1] Micromoles/100 ml blood plasma; S 1 sample taken day 1; S 2 sample taken day 3.

TABLE 2

Total[1] free amino acids of dog plasma[2] and gut contents[3]

Dogs[4]	Fasting	2-Hour postfeeding				
	Saphenous vein	Jugular vein	Carotid artery	Mesenteric vein	Portal vein	Gut contents
Group 1						
Dog 4 (F 16)	164	217	246	278	348	223
Dog 6 (F 20)	200	237	214	246	246	224
Dog 3 (M 13)	298	372	308	524	396	422
Group 2						
Dog 2 (M 19)[5]	375	309	267	278	270	446
Dog 5 (M 15)	372	225	239	312	241	435

[1] The amino acids included in these summations are those listed in tables 3 and 4.
[2] Micromoles/100 ml blood plasma.
[3] Micromoles/30 mg NPN (approximate NPN/100 ml plasma).
[4] M = male; F = female; numbers in parentheses indicate weight in kilograms.
[5] Same animal used in experiment A.

total free amino acids in the different plasma samples from each dog are shown in table 2. It is unfeasible to measure volume of the semi-solid gut contents and hence amino acids in gut contents are expressed on the basis of 30 mg of NPN, the approximate nonprotein nitrogen of 100 ml of dog plasma. The relative molar ratios of the amino acids in blood and gut contents are recorded in tables 3 and 4. Since leucine appeared to fluctuate less than the other essential amino acids all molar ratios are based on leucine concentration as unity.

Ethanolamine was absent from fasting blood but present in post-feeding blood of

TABLE 3

Relative concentrations of free amino acids in dog blood plasma and gut contents (exp. B, group 1) (leucine = 1.0)[1]

Amino acid	Fasting	2-Hour postfeeding				
	Saphenous vein	Jugular vein	Carotid artery	Mesenteric vein	Portal vein	Gut contents
Arginine	0.9	0.6	0.5	0.7	0.4	1.0
Histidine	0.9	0.5	0.5	0.5	0.6	0.3
Isoleucine	0.9	0.8	0.9	0.8	0.9	0.8
Leucine	1.0	1.0	1.0	1.0	1.0	1.0
Lysine	1.6	1.1	1.0	1.1	1.0	1.4
Methionine	0.4	0.2	0.2	0.2	0.2	0.2
Phenylalanine	0.8	0.5	0.5	0.7	0.5	1.0
Threonine	3.9	1.9	1.7	1.8	1.8	0.8
Tryptophan	0.2	0.2	0.4	0.2	0.2	0.1
Valine	1.9	1.6	1.9	1.6	1.8	0.8
Alanine	5.1	4.4	2.8	4.2	4.6	1.3
Aspartic acid	0.0	0.0	0.0	0.0	0.0	0.3
Glutamic acid	0.7	0.4	0.4	0.8	0.5	0.8
Glycine	3.4	1.4	1.0	1.5	1.5	0.9
Proline	1.9	0.7	0.7	0.9	1.0	0.5
Serine	2.1	0.9	0.6	0.9	0.8	0.7
Tyrosine	0.5	0.6	0.3	0.5	0.4	0.9

[1] Average leucine value: (a) μmole/100 ml plasma — saphenous vein, 8.4; jugular vein, 16.3; carotid artery, 17.9; mesenteric vein, 19.8; portal vein, 19.1; (b) μmole/30 mg NPN — gut contents, 22.2.

TABLE 4

Relative concentrations of free amino acids in dog blood plasma and gut contents (exp. B, group 2) (leucine = 1.0)[1]

Amino acid	Fasting	2-Hour postfeeding				
	Saphenous vein	Jugular vein	Carotid artery	Mesenteric vein	Portal vein	Gut contents
Arginine	0.6	0.4	0.5	0.7	0.6	0.8
Histidine	0.7	0.7	0.7	0.5	0.6	0.3
Isoleucine	0.9	1.0	0.9	0.9	0.9	0.5
Leucine	1.0	1.0	1.0	1.0	1.0	1.0
Lysine	1.4	1.3	1.3	1.0	1.6	1.1
Methionine	0.3	0.3	0.3	0.3	0.3	0.1
Phenylalanine	0.8	0.6	0.9	0.6	0.8	0.8
Threonine	3.0	3.5	2.9	2.1	2.3	0.4
Tryptophan	0.2	0.0	0.2	0.1	0.2	0.1
Valine	1.8	1.8	1.6	1.5	1.3	0.7
Alanine	4.6	4.6	3.7	4.2	3.8	1.1
Aspartic acid	0.0	0.0	0.0	0.0	0.0	0.1
Glutamic acid	0.5	0.6	0.5	0.5	0.9	0.7
Glycine	2.0	1.7	1.4	1.2	1.1	0.6
Proline	1.2	1.2	1.1	0.9	1.3	0.3
Serine	1.0	1.1	1.0	0.6	0.7	0.5
Tyrosine	0.5	0.4	0.3	0.5	0.7	0.6

[1] Average leucine value: (a) μmole/100 ml plasma — saphenous vein, 18.2; jugular vein, 13.2; carotid artery, 13.8; mesenteric vein, 17.6; portal vein, 14.1; (b) μmole/30 mg NPN — gut contents, 44.7.

all animals. Citrulline was absent from gut contents but always present in blood. Cysteic acid (cysteine), ornithine and taurine, were either absent from or present in blood in very small amounts. Asparagine content ranged from 46 to 141 μmole/100 ml of fasting plasma and 39 to 148 after a meal. Average values for protein and nonprotein nitrogen in plasma of fasting dogs were 6.4 gm and 28.8 mg/100 ml. Average values for K and Na were 3.3 and 138.0 mEq/liter. Ingestion of test meals failed to alter these ratios significantly.

DISCUSSION

The results of these experiments indicate that total free amino acid concentration in blood after feeding may increase or decrease. When increases occurred they were evident in all blood samples but they were greatest in mesenteric and portal blood. Amino acids being absorbed from the gut lumen pass through the mucosa, which may remove some of them, and into the mesenteric and portal veins and are then subject to selection by the liver before entering the peripheral vessels. In group 2 the total free amino acid concentration decreased as a result of feeding. With the possible exception of the mesenteric vein, no differences in total amino acid were evident among the blood samples taken from the various vessels after feeding. Ebb and flow of plasma amino acids occur in the same animal. Dog 2, in the first two fasting periods, yielded low values (table 1) but in the third fasting period the values decreased in the high group (table 2). The amount of amino acids in the circulation is probably mainly the resultant of protein synthesis and degradation. Some losses occur by secretion in the kidney and deamination in the liver but the dominating influence on plasma amino acids must be the dynamic state of protein metabolism.

What acute change in protein metabolism might, as the result of feeding, cause a decrease in circulating amino acids? Two hours after feeding, most of the digestive processes are operating at high intensity. Salivary, gastric, pancreatic, hepatic and intestinal secretory cells have discharged copious volumes of digestive juices which contain enzymes and other proteins. If extensive neosynthesis of these proteins should occur simultaneously in all glands, plasma amino acids might conceivably fall, despite a simultaneous influx from the gut. Digestive glands vary greatly from day to day in response to the feeding of identical test meals. This may explain in part the unpredictable behavior of plasma amino acids after feeding.

Despite variations in amounts of amino acids in plasma samples, the molar ratios remained fairly constant (tables 3 and 4). The body apparently tends to maintain a relatively uniform mixture of free amino acids in plasma during periods of high-quality protein intake. The molar ratios in plasma do not necessarily correspond to those in gut contents. This is perhaps not unexpected because the gut mucosa itself may remove some amino acids as they are being absorbed (Ju and Nasset, '59). The data give evidence of the mucosal transamination reactions that affect the concentrations of alanine, glutamic acid and aspartic acid on the two sides of the membrane (Neame and Wiseman, '57).

SUMMARY

Dogs were fasted 24 hours before being fed 200 gm of ground lean beef. Total free amino acids in fasting plasma varied from about 200 to 400 μmole/100 ml of plasma. When fasting values were high, feeding reduced them and vice versa. Regardless of total amino acids there was little difference in molar ratios. Plasma amino acid molar ratios may differ from the molar ratios of free amino acids in gut contents.

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Influence of Caloric Level and Protein Quality on the Manifestations of Protein Deficiency in the Young Pig[1]

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Knowles ('57) and Heard et al. ('58) observed in the young pig that the addition of extra calories, as carbohydrate, to a low-protein diet precipitated a condition resembling kwashiorkor in humans. Wheat gluten was the source of protein fed both groups of pigs. Among the changes observed on autopsy of humans suffering from kwashiorkor as reported by Follis ('58) were accumulation of fluid in the subcutaneous tissues and in the peritoneal, pleural and pericardial cavities, decreased size and activity of the pancreatic acinar cells, hyalinization of the glomeruli in the kidneys, atrophy of the intestinal mucosa, thyroid gland, adrenal cortex and salivary glands and fatty infiltration of the liver.

This study was conducted to observe the effect of the addition of extra calories, as fat, to diets of ad libitum-fed pigs on the development of protein deficiency symptoms, to observe the effect of protein quality on the response to extra calories, and to compare the symptoms obtained with those observed in human kwashiorkor.

EXPERIMENTAL

Twenty Yorkshire pigs weighing an average of 6.3 kg were divided on the basis of weight into 5 outcome groups of 4 pigs each. They were housed in concrete-floor pens and given feed and water ad libitum. Four groups were fed the diets shown in table 1. These were purified diets (5% protein) in which wheat gluten or casein served as the protein source each fed at two levels of fat (3% or 23% corn oil). The fifth group served as the positive control and received a typical stock diet with the following composition: ground yellow corn, 40.5; rolled oats, 16.5; soybean meal, 22.0; fish meal, 5.0; dried brewer's yeast, 1.0; trace-mineralized salt, 0.5; dicalcium phosphate, 1.5; sucrose, 10.0; stabilized lard, 2.5; vitamin supplement (to supply 1.5 times the estimated requirement of fat-and water-soluble vitamins), 0.5%. The 4 purified diets were stored under refrigeration until use. Fresh feed was added to the feeders daily.

Individual pigs were weighed weekly and feed consumed by each group of pigs was recorded weekly. All pigs were given 100 mg of iron intramuscularly (as iron dextran) during week 7. Blood samples from all pigs were obtained from the anterior vena cava during weeks 6, 8, 10, 12 and 14. Total serum proteins (Gornall, '49) were determined on samples from weeks 6, 10 and 14 and electrophoretic patterns were run on the same samples using the Spinco Model R paper electrophoresis system. Hemoglobin (Sanford and Sheard, '29) and hematocrit (Wintrobe, '47) values were determined on samples from weeks 8 and 12. Positive control pigs were removed from the trial at the end of the thirteenth week. Rectal temperature was determined on all pigs fed purified diets on two successive days during week 14. The pigs fed purified diets were killed by intraperitoneal injection of sodium pentobarbital and autopsied at the end of the fourteenth week. Gross pathological conditions were observed and adrenal, heart, kidney, liver, spleen and

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TABLE 1

Composition of low-protein diets

Protein source	Casein		Wheat gluten	
Level of fat, %	3	23	3	23
	%	%	%	%
Dextrin	25.0	25.0	25.0	25.0
Glucose[1]	57.1	37.1	57.0	37.0
Casein[2]	5.9	5.9	—	—
Wheat gluten[3]	—	—	6.0	6.0
Corn oil[4]	3.0	23.0	3.0	23.0
Cellulose[5]	4.0	4.0	4.0	4.0
Minerals[6]	5.0	5.0	5.0	5.0
Vitamins[7]	+	+	+	+
Total	100.0	100.0	100.0	100.0

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] "Vitamin-Free" Casein, Nutritional Biochemicals Corporation, Cleveland.
[3] Purified, General Biochemicals, Inc., Chagrin Falls, Ohio.
[4] Mazola, Corn Products Company, Argo, Illinois.
[5] Solka Floc, Brown Company, Boston 14, Massachusetts.
[6] Composition, gm/kg of diet: $CaHPO_4 \cdot 2H_2O$, 15.40; $CaCO_3$, 12.32; KH_2PO_4, 17.16; NaCl, 6.16; $CuSO_4$, 0.57; $FeSO_4$,0.66; $MnSO_4$, 0.185; $ZnSO_4$, 0.343; MgO, 0.818; $CoCl_2$, 0.011; KI, 0.0004.
[7] Composition, units/kg of diet: vitamin A, 2,640 IU; vitamin D, 330 IU; thiamine·HCl, 1.98 mg; riboflavin, 4.84 mg; niacin, 33.0 mg; Ca-D-pantothenate, 19.8 mg; pyridoxine·HCl, 1.65 mg; inositol, 1498 mg; *p*-aminobenzoic acid, 147.4 mg; folic acid, 1.41 mg; α-tocopheryl acetate, 99.0 mg; menadione, 3.30 mg; vitamin B_{12}, 33 μg; choline chloride, 1320 mg.

thyroid weights were recorded. For histopathology, sections were taken from the stomach, liver, pancreas, heart, lungs, kidneys, proximal tibial epiphysis, brain, spleen, bone marrow, skin, pituitary, thyroid, adrenal, ovaries, testes. Soft tissues were fixed in Bouin's solution, bones in buffered formalin. Paraffin-embedded tissues were sectioned at 6 μ, frozen tissues at 10 μ. Stains were oil red O for fat on frozen sections and for embedded sections, hematoxylin-eosin, Cresyl-Echt-Violet for Nissl substance, Weil-Weigerts myelin stain for myelin tracts of brain, Toluidine blue for metachromatic granules.

Liver fat content was determined by ether extraction; bone ash was determined by drying for 48 hours at 70°C, extracting with ether for 48 hours and ashing overnight at 550°C. The width of the proximal tibial epiphysis was determined by taking the average of 10 measurements obtained with a micrometer.

RESULTS

The pigs fed diets containing casein gained weight at approximately the same rate at both levels of fat until week 8 when those receiving the high-fat diet reached a plateau. The low-fat group continued to gain throughout the 14 weeks of experiment. In contrast, the pigs fed diets containing wheat gluten failed to gain weight appreciably during the 14 weeks, in the presence of either a low or high level of fat. The body weight curves are shown in figure 1.

The animals fed the positive control diet gained an average of 640 gm daily and were removed from the experiment after 13 weeks (due to insufficient floor space), weighing an average of 64.6 kg.

The amount of feed consumed by pigs fed diets containing casein increased steadily during the first 6 weeks at both levels of fat. From week 6 to week 14 the feed consumption of pigs fed the low level of fat continued to increase, whereas that of pigs fed the high level steadily declined. This resulted in a decline in daily protein intake to 15.9 gm during the final two weeks among pigs in the latter group as compared with 68.7 gm in the former. The first 6 weeks involved a greater daily caloric consumption in the group fed high fat followed by a gradual reduction during the final 8 weeks. Feed consumption continued to increase throughout the 14 weeks of the experiment in the group fed the low level of fat. The feed consumed by pigs fed diets containing gluten gradually declined throughout the 14 weeks of the experiment at both levels of fat. During the final two weeks the protein intake of pigs fed gluten was approximately 8% of National Research Council ('59) recom-

mendations for animals of this size. The caloric intake of these animals remained above NRC ('59) recommendations during the first 8 weeks and then dropped below. During the final two weeks the caloric intake was approximately 50% of the recommended level (NRC, '59).

Vitamin and mineral intakes were above NRC ('59) recommendations for all groups during the 14 weeks of the experiment, except during the final 4 weeks for pigs fed the wheat gluten, high-fat diet. These pigs consumed less than 200 gm of feed/day during the final 4 weeks. Data on feed consumption are given in figure 2.

The animals fed the wheat gluten diets became emaciated and feeble during the final two weeks. Hair coats were rough and intermittent diarrhea occurred during this period. Rectal temperatures were lower ($P < 0.05$) during week 14 in the animals fed the wheat gluten-high fat diet than in those fed the other three purified diets (101.4°F vs. 102.9, 102.4 and 102.8°F).

The total serum protein was greater ($P < 0.01$) in the pigs fed the positive control diet than in those fed the other 4 diets at 6 and 10 weeks. Even at 14 weeks no significant differences existed among the pigs fed any of the 4 purified diets despite the striking differences in growth and feed consumption. Total serum protein tended to reach a plateau in all groups from week 10 to 13, indicating that a certain minimum level is maintained even in severe protein depletion. Electrophoretic patterns showed that the albumin fraction was chiefly responsible for the drop in total serum protein observed in all groups. Both the percentage and the absolute amount of albumin decreased in all 4 groups of pigs fed the low-protein diets ($P < 0.01$). The change in total globulins was significant ($P < 0.01$) when expressed as a percentage of the total protein, but not when expressed in absolute amounts. The α-, β-, and γ-globulin components remained constant throughout the experiment when expressed as grams per 100 ml, but increased when expressed as a percentage of the total serum protein. The alpha$_1$ fraction had disappeared by week 10 in all but the pigs fed the positive control ration. The data on serum protein are presented in table 2.

The blood hemoglobin and hematocrit levels of pigs fed wheat gluten were sig-

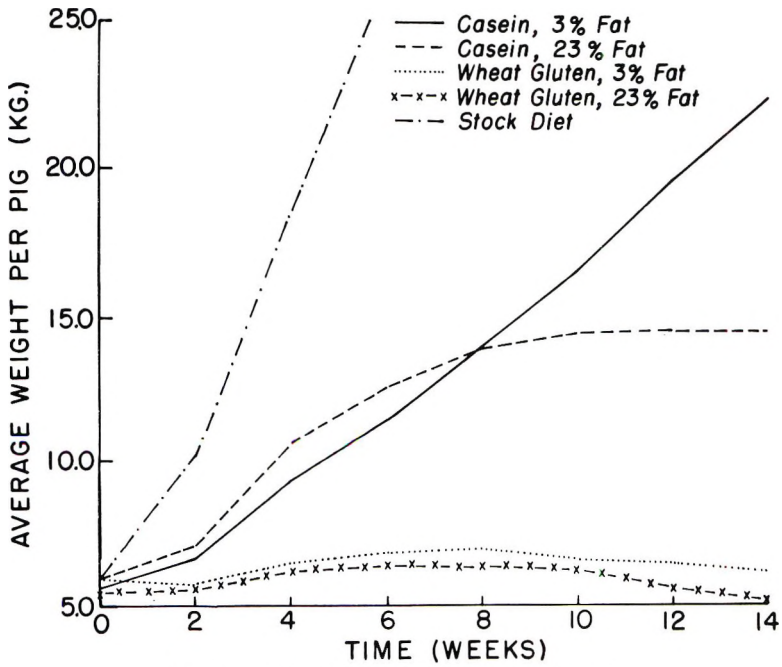

Fig. 1 Effect of diet on body weight gain.

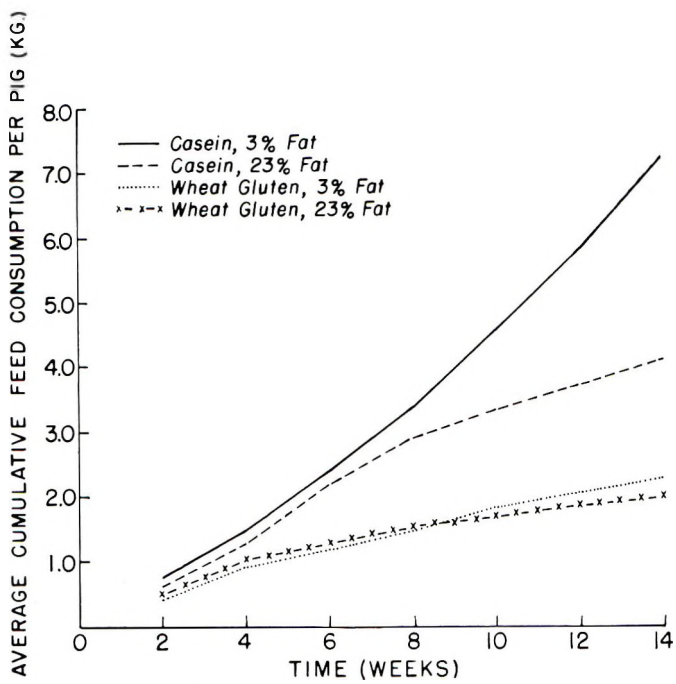

Fig. 2 Effect of diet on feed consumption.

TABLE 2

Effect of level of dietary energy and source of protein on blood serum proteins

Protein source	Control[1]	Casein		Wheat gluten	
Level of fat, %	—	3	23	3	23
Week 6[2]					
Total, gm/100 ml	7.40[3]	4.80	5.48	4.28	4.23
Albumin, gm/100 ml	3.32[3]	1.01	1.56	0.72	0.91
Albumin:globulin	0.82[3]	0.28	0.42	0.21	0.27
Week 10[2]					
Total, gm/100 ml	7.30[3]	4.45	4.30	3.90	4.02
Albumin, gm/100 ml	3.67[3]	0.90	0.86	0.58	0.60
Albumin:globulin	1.01[3]	0.26	0.25	0.17	0.19
Week 14[2]					
Total, gm/100 ml	—	4.98	4.58	4.08	4.10
Albumin, gm/100 ml	—	0.81	0.68	0.32	0.46
Albumin:globulin	—	0.19	0.18	0.09	0.13

[1] 18% protein from natural feedstuffs (see text).
[2] Mean of 4 pigs/group.
[3] Significantly greater than all other treatments ($P < 0.01$).

nificantly lower ($P < 0.01$) at both 8 and 12 weeks than those of pigs fed casein. Pigs fed the high level of fat and wheat gluten had lower hemoglobin and hematocrit levels ($P < 0.05$) at 12 weeks than those fed the other three purified diets, while those fed the positive control diet had higher levels ($P < 0.01$) of both constituents than pigs fed any of the purified diets. This could not be related to differences in dietary iron intake since all pigs were given iron intramuscularly during week 7. The data on hemoglobin and hematocrit are presented in table 3.

No significant differences due to diet were found in kidney, thyroid or liver

TABLE 3

Effect of level of dietary energy and source of protein on blood hemoglobin and hematocrit

Protein source	Control[1]	Casein		Wheat gluten	
Level of fat, %	—	3	23	3	23
Week 8[2]					
Hemoglobin, gm/100 ml[3]	11.5	9.6	9.6	7.1	7.9
Week 12[2]					
Hemoglobin, gm/100 ml[4]	12.9	10.3	10.1	7.6	6.3
Hematocrit[5]	40.7	30.3	28.5	26.3	18.8

[1] 18% protein from natural feedstuffs (see text).
[2] Mean of 4 pigs/group.
[3] Control significantly greater than casein ($P < 0.05$) and gluten ($P < 0.01$).
[4] Control significantly greater than casein and gluten ($P < 0.01$). Casein significantly greater than gluten ($P < 0.01$). Gluten, 3% fat significantly greater than gluten, 23% fat ($P < 0.05$).
[5] Control significantly greater than casein and gluten ($P < 0.01$). Casein, both levels of fat and gluten, 3% fat significantly greater than gluten, 23% fat ($P < 0.05$).

TABLE 4

Effect of level of dietary energy and source of protein on weight of organs

Protein source	Casein		Wheat gluten	
Level of fat, %	3	23	3	23
	Percentage of live body weight[1]			
Adrenals × 100[2]	0.77	0.82	1.74	2.43
Heart[3]	0.43	0.48	0.63	0.68
Kidneys	0.37	0.33	0.38	0.42
Liver	2.59	2.37	3.05	3.40
Spleen[4]	0.14	0.13	0.11	0.10
Thyroid × 100	0.79	1.06	1.03	1.13

[1] Each value is mean of 4 pigs.
[2] Gluten significantly greater than casein ($P < 0.01$). Gluten, 23% fat significantly greater than gluten, 3% fat ($P < 0.05$).
[3] Gluten significantly greater than casein ($P < 0.01$).
[4] Casein significantly greater than gluten ($P < 0.05$).

weight expressed as a percentage of live body weight. There was a trend, however, for liver weights of pigs fed wheat gluten to be larger than those of pigs fed casein. Adrenal glands from pigs fed wheat gluten were significantly heavier ($P < 0.01$) than those from pigs fed casein. Of the pigs fed wheat gluten, those receiving the high level of fat had larger adrenals ($P < 0.05$) than those fed the low level. The weight of the heart was greater in pigs fed wheat gluten than in those fed casein ($P < 0.01$). No difference was observed in heart weight due to caloric level. The only significant difference in spleen weights occurred between the casein-low fat diet and the two wheat gluten diets ($P < 0.05$), the former being heavier. The data on organ weights are presented in table 4.

The ash content of the dry, fat-free bone was lower in pigs fed wheat gluten than in those fed casein ($P < 0.01$). The width of the proximal tibial epiphysis was significantly ($P < 0.05$) less in the pigs fed wheat gluten-high fat than in those fed casein-low fat. The average values for pigs fed casein were higher than those for pigs fed wheat gluten, at either level of fat, although these differences did not reach statistical significance. The fat content of livers from pigs fed wheat gluten tended to be higher than that from those fed casein, although the differences were not statistically significant ($P > 0.05$). The data on bone ash, epiphyseal width and liver fat are presented in table 5.

Gross pathology

Pigs fed wheat gluten-high fat exhibited a pronounced general anemia and diffuse moderate subcutaneous edema. Serous atrophy of fat deposits and bone marrow was advanced. Bones were brittle and transudation to the serous cavities oc-

TABLE 5

Effect of level of dietary energy and source of protein on bone ash, epiphyseal width and liver fat

Protein source	Casein		Wheat gluten	
Level of fat, %	3	23	3	23
Bone ash, %[1]	52.1	52.2	48.3	47.5
Width of epiphysis, micra[2]	662.5	549.1	428.4	301.9
Liver fat, %[3]	11.6	16.8	22.1	24.1

[1] Tibia, dry, fat-free. Each value is mean of 4 pigs. Casein significantly greater than gluten ($P < 0.01$).
[2] Tibia, proximal. Each value is mean of 10 measurements taken on each of 4 pigs. Casein, 3% fat significantly greater than gluten, 23% fat ($P < 0.05$).
[3] Dry basis. Each value is mean of 4 pigs. No significant differences.

curred in 3 of the 4 pigs. The group fed wheat gluten-low fat showed the same gross anatomy, but with less severe anemia, subcutaneous edema, and serous atrophy in the bone marrow. Moderate ascites occurred but no transudation to other serous cavities was observed in this group. Pigs fed high fat-casein showed only mild anemia and ascites and bones were longer and thicker than those of pigs fed gluten. Those fed casein-low fat showed normal gross anatomy.

Microscopic pathology

Stomach. In the high fat-wheat gluten group, surface epithelium was markedly atrophic with no mucigeneous granules. The foveolae were very shallow and glands of the mucosa presented the classical picture of "signet-ring cells." There was extensive edema in the muscle layers and subserosa. The same changes were noted in the low fat-wheat gluten and high fat-casein groups but less severe. The low fat-casein group appeared normal.

Duodenum. Villi were markedly atrophic in both the high and low fat-wheat gluten groups. Goblet cells were numerous and mucin content was abundant. Only slight changes were noted in the high fat-casein group and the low fat-casein group appeared normal.

Pancreas. The two zones normally present in the exocrine epithelium could not be distinguished in the high fat-wheat gluten group. In this group the endocrine portion of the gland was markedly atrophic and few islets were seen. Similar but less severe changes occurred in the low fat-wheat gluten group. Both the high and low fat-casein groups showed less atrophy than the other groups, but granulation appeared only in the low fat-casein group.

Heart. Sarcoplasm was less abundant than normal in the high and low fat-wheat gluten groups and the high fat-casein group with cross-striations poorly marked or absent. Moderate interstitial edema was present. Purkinje fibers were swollen and presented vacuoles in the sarcoplasm. The low fat-casein group appeared normal.

Skeletal muscle. Extreme atrophy and partial or complete loss of cross-striations, with edema between the fibers occurred in both the high and low fat-wheat gluten groups. The high and low fat-casein groups appeared normal.

Lung. There was extreme atrophy of the bronchiolar and bronchial epithelium and a change from columnar to cuboidal shape in both the high and low fat-wheat gluten groups. Goblet cells were more numerous than normal and mucin retention occurred with the formation of "signet-ring cells." Areas of atelectosis were observed which resulted from a swelling of the alveolar lining cells and from edema. Both the high and low fat-casein groups appeared normal except for some atrophy of the epithelium in the former.

Kidney. There was pronounced intraglomerular edema and extensive atrophy of capsular epithelium of the glomeruli in the high and low fat-wheat gluten groups. Hydropic degeneration occurred in the tubular and renal pelvis epithelium of the latter. Edema was less in the high fat-casein group but tubular epithelium and proximal convoluted tubules showed some degenerative changes. Slight vascular degeneration in the proximal convoluted tubules and pelvic epithelium were the only changes in the low fat-casein group.

Proximal tibial epiphysis. In the high and low fat-wheat gluten groups and the high fat-casein group there was very little activity. In both wheat gluten groups the epiphysis consisted of about two-thirds resting cartilage. Trabeculae were few and small and not lined by osteoblasts. No osteoclastic activity was present. In the high fat-casein group about one-third of the epiphysis was resting cartilage and more trabeculae and slight osteoclastic activity were observed. Only in the high fat-casein group was there any osteoid present.

Brain. Drastic changes occurred in the high fat-wheat gluten group. There was a reduction in numbers of neurons in all grey matter, especially in the thalamic and medullar nuclei and in the Purkinje cells of the cerebellum. Neurons were swollen with poorly marked axons and dendrites. Nissl substances were limited or absent. Moderate edema was present as indicated by widened Virchow-Robin spaces. Similar but less severe changes were observed in the low fat-wheat gluten, high fat-casein and low fat-casein groups in descending order of severity.

Spleen. White and red pulp were atrophic in the high and low fat-wheat gluten groups, the red pulp appearing as an empty network of reticular cells. The capsules and trabeculae were more predominant in the above groups than in the high and low fat-casein groups. Walls of the arteries in these enlarged trabeculae were hyalinized. Hemosiderin was present only in the low fat-casein group.

Bone marrow. Serous atrophy observed grossly in the high and low fat-wheat gluten group was verified. Cellular elements were greatly reduced. The erythropoietic series was most affected. While the high and low fat-casein groups contained many cells, both exhibited hypoplasia.

Skin. There was extreme atrophy of the epidermis in the high fat-wheat gluten group with the layers being indistinguishable. Cells were elongated and flattened and the stratum corneum was present only as a thin layer. Edema was present in the subcutis. The low fat-wheat gluten and high fat-casein groups were similar but less severe and a deep and superficial layer could be distinguished. All layers were easily distinguished in the low fat-casein group. Hair papillae were few and markedly atrophic in the high fat-wheat gluten group and the cells of the skin glands were atrophic and lined a large empty lumen. The low fat-wheat gluten and high fat-casein groups showed similar but less severe changes in the hair papillae while the low fat-casein group appeared normal.

Pituitary. Oesinophilic cells were few and irregularly distributed in the high and low fat-wheat gluten groups. Similar but less severe changes occurred in the high fat-casein group while the low fat-casein group appeared normal.

Thyroid. The activity was lowest in the high and low fat-wheat gluten groups and highest in the low fat-casein group.

Adrenals. The cortex contained abundant fat in the high and low fat-wheat gluten groups and the zona glomerulosa in the former. Lesser amounts, but greater than normal, were found in the high and low fat-casein groups.

Ovaries. Many primary follicles were present in all groups but anticum formation occurred only in the high and low fat-casein groups (two females in the high and low fat-wheat gluten groups and three in the high and low fat-casein groups).

Testes. Activity was present in only one animal (in the high fat-casein group).

DISCUSSION

These results demonstrate the similarity in biochemical and anatomical changes associated with protein deficiency in pigs and kwashiorkor in humans. Dean and Schwartz ('53), Cravioto ('58) and Baptist et al. ('59) reported reduced serum albumin levels and reduced albumin: globulin ratios in human subjects with kwashiorkor. These observations agree with those of present study. It is of interest that total serum protein level did not change appreciably after the sixth week of the present study, even among pigs consuming wheat gluten to supply less than 10 gm of protein daily during the latter one-half of the experiment. This was due to the persistency of the level of the α-, β-, and γ- globulin fractions. The serum albumin

and globulin levels observed in this study are in close agreement with those of Knowles ('57) and Heard et al. ('58), who fed 4.5 or 6.5% wheat protein diets to pigs, but somewhat lower than values reported by Cartwright and Wintrobe ('48). That hemoglobin and hematocrit levels were maintained less efficiently than serum globulins in severe protein deficiency was shown among all pigs fed low-protein diets, but especially those fed wheat gluten-high fat diets. Feed intake and, consequently, protein intake was inversely related to caloric density so that pigs fed the casein or wheat gluten-high fat diets developed more severe protein deficiency symptoms than those consuming the corresponding low fat diets. A similar effect of energy level on the growth response in pigs fed low-protein diets has been observed previously (Barnes et al., '59). Quality of protein exerted a pronounced effect on this relationship, the pigs fed casein showing less marked aggravation of protein deficiency in the presence of extra calories from added fat than those fed wheat gluten.

The effect of protein deficiency on the weight of the spleen and adrenal gland agrees with the observation of McCance ('60) among pigs maintained for 9 to 14 months at a weight of 4 to 5 kg. The diets of animals in that study were restricted in energy as well as in protein. The increased adrenal weights in the pigs fed wheat gluten in the present study appeared to be associated with greater fat deposition in the cortex. The lesser spleen weights in these same animals was probably a reflection of a limited storage of erythrocytes when the wheat gluten diets were fed. The greater heart weight among the pigs fed gluten was probably a manifestation of the greater depletion of body nutrients other than those in vital organs in this group than in the casein-fed pigs.

The lower ash content of the tibia in gluten-fed pigs than in casein-fed pigs corroborates the reports of Murthy et al. ('55) and Joseph et al. ('59) who indicated that calcium and phosphorus retention were decreased in humans with kwashiorkor. Growth of long bones as indicated by the width of the proximal tibial epiphysis was related to both protein source and energy level, the pigs fed the casein-low fat diet and those fed the gluten-high fat diet representing the fastest and slowest growth, respectively. It is significant that while both the high- and low-fat groups fed wheat gluten maintained essentially constant weight over the entire experimental period, pigs in the latter group showed greater bone growth and were less emaciated than those in the former. The lower hematocrit of the high-fat group at week 12 indicates that the maintenance of body weight was at least partially a result of greater edema.

The increase in liver fat in pigs fed wheat gluten agrees with the reports of Williams ('35), Scrimshaw et al. ('56) and Waterlow and Wills ('60) in association with kwashiorkor in humans.

The pathological findings, both gross and microscopic, demonstrated the greater effect of wheat gluten than casein and of high fat than low fat on the changes in the fat deposits, bone marrow, bone, stomach, liver, pancreas, heart, lungs, kidneys, brain, spleen, skin, pituitary, thyroid, adrenals, ovaries, testes. The severity of these changes was inversely related to the absolute amounts of protein consumed by each group.

The anatomical and biochemical changes observed in the young pig fed protein-deficient diets closely resemble those present in chronic protein deficiency (kwashiorkor) in the human infant.

SUMMARY

Young pigs (approximately three weeks old, average weight 6.3 kg) were used to study the effect of caloric density and protein quality of the diet on the biochemical and anatomical manifestations of protein deficiency. Purified diets containing 5% of protein from casein or wheat gluten and either 3 or 23% fat (corn oil) were fed ad libitum for 14 weeks. A stock diet containing 18% of protein served as the positive control.

Casein produced a 182-gm average daily gain in the presence of 3% of fat. Weight gain was similar during the first 8 weeks when casein was fed in the presence of 23% of fat, but weights reached a plateau and remained constant during the remainder of the trial. Wheat gluten failed

to support body weight gain at either level of fat.

Deficiency symptoms noted were similar to those reported in kwashiorkor in humans. Symptoms were most severe in pigs fed wheat gluten with 23% of fat, and least severe in those fed casein with 3% of fat. The pigs in the former group were emaciated and feeble during the last two weeks, had reduced rectal temperature ($P < 0.01$) and lower hematocrit ($P < 0.01$) than the other groups. Hemoglobin was depressed ($P < 0.01$) with wheat gluten as compared with casein, but total serum protein declined to about 4 gm/100 ml after 6 weeks in all groups and plateaued. Total globulins remained constant in all groups throughout the experiment.

Adrenal, heart and liver weghts (as percentage of body weight) were greater in pigs fed wheat gluten than in those fed casein. Bone growth was depressed, especially in gluten-fed pigs, and carcasses showed extensive edema throughout. Pathological changes occurred in all organs and tissues studied, being the most severe in the pigs fed gluten with 23% fat and least severe in those fed casein with 3% fat. Both caloric density and protein quality exerted an effect on the severity of protein deficiency symptoms in the pig. The severity of symptoms was inversely related to the absolute amounts of protein voluntarily consumed daily by each group.

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