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Weight		*Length*	
kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
		μμ	micromicron
Volume			
m^3	cubic meter		
cm^3	cubic centimeter	*Area*	
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

Symbols. When preceded by a figure, the following symbols are used:

A	Angstrom units	°	degree
%	per cent	ppm	parts per million

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The Thiamine Requirement of the Mink for Growth and Fur Development[1]

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Although a number of papers have been published on the nutritional requirements of the mink, studies on the quantitative requirement of the mink for the individual vitamins are limited. Basset and coworkers (Basset, Loosli and Wilke, '48; Basset, Harris and Wilke, '51), using semi-practical ranch diets, have presented data on the requirement of the mink for vitamin A and vitamin D. Schaefer, Whitehair and Elvehjem ('46) were the first workers to employ purified diets in the study of mink nutrition. Their initial experiments were concerned with the folic acid requirement of the mink. By the use of this purified diet, Leoschke, Lalor and Elvehjem ('53) were able to determine the vitamin B_{12} requirement of the mink.

The development of a purified diet capable of supporting normal growth and fur production of the mink has only recently been attained (Leoschke and Elvehjem, '59). This achievement has greatly facilitated the study of the vitamin requirements of the mink. The data presented in this report are concerned with the thiamine requirement of the mink for growth and fur development.

EXPERIMENTAL AND RESULTS

The composition of the purified basal diet used in this study is as follows: sucrose 54.25, casein[3] 25, cottonseed oil 6, lard 5, cellulose[4] 5, salts IV (Phillips and Hart, '35) 4, L-arginine·HCl 0.5 and DL-methionine 0.25%. Each 100 gm of ration were supplemented with 0.2 mg pyridoxine·HCl, 0.4 mg riboflavin, 1.5 mg Ca pantothenate, 4.0 mg nicotinic acid, 100 mg choline·HCl, 25 mg *i*-inositol, 50 mg *p*-aminobenzoic acid, 0.5 mg 2 methyl, 1-4 napthoquinone, 0.1 mg pteroylglutamic acid, 0.025 mg biotin and 0.004 mg of vitamin B_{12}. Haliver oil fortified with α-tocopherol acetate and vitamin D_3 was added so that 100 gm of ration contained 1,200 I.U. vitamin A, 120 I.U. vitamin D and 4 mg of α-tocopherol acetate. Feed consumption of the mink on the purified diet varied from 75 to 150 gm per animal per day. Graded levels of thiamine·HCl were mixed with this purified basal diet three times a week. This procedure was followed in order to minimize possible destruction of the vitamin during storage. All diets were stored in a refrigerator prior to feeding to the animals.

The experimental feeding period extended from early July to late October. This period encompasses the late growth and fur production phases of the mink's life. Weanling male mink kits weighing from 400 to 800 gm were fed the purified basal diet supplemented with 0.2 mg of thiamine·HCl per 100 gm of ration for 10 days. After the preliminary period of adjustment to the purified diet, the mink were divided on the basis of weight and colorphase into 5 groups of 14 mink each. The experimental diets consisted of the purified basal diet supplemented with 0 (group I), 60 (group II), 90 (group III), 120 (group IV) and 150 μg (group V) of thiamine·HCl per 100 gm of ration.

Growth of the mink on the experimental diets containing graded levels of thiamine·HCl is shown in figure 1. The weight data are presented for each group to the point of initiation of paralysis and death of

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We wish to acknowledge our indebtedness to Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, for the crystalline vitamins.

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[3] General Biochemicals, Inc.

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

mink within the group. At the end of one week a significant reduction of feed intake was noted in group I (0 thiamine supplementation). Within three weeks all of the mink in this group had developed the typical symptoms of a thiamine deficiency and died. These symptoms included anorexia, loss of weight, lack of muscle coordination, extreme weakness and finally paralysis and death. The deficiency symptoms are similar to those noted in the first observations of thiamine deficiency in fur-bearing animals on record, the study of Green, Carlson and Evans ('41) on the Chastek Paralysis disease of foxes. By the end of the 10-week experimental period, 8 of the mink in group II and three of the mink in group III had died as a result of thiamine deficiency. None of the mink in groups IV and V manifested symptoms of a thiamine deficiency. Figure 2 illustrates the paralysis seen in final stages of the thiamine deficiency. Poor quality fur was noted on those mink in groups II and III which had shown suboptimal growth during the experimental period. The fur of the mink in groups IV and V was similar and of good quality.

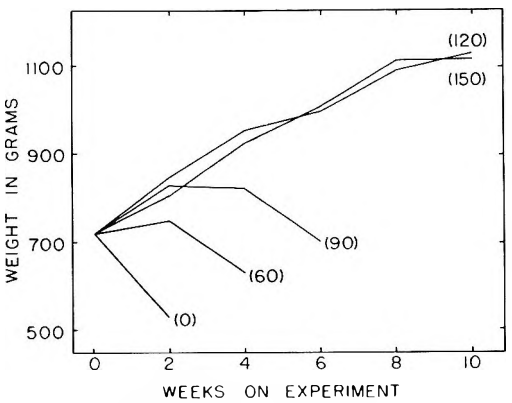

Fig. 1 Growth of mink fed a purified basal diet containing graded levels of thiamine·HCl. The number within parentheses indicates the micrograms of thiamine·HCl added per 100 gm of basal diet.

Fig. 2 Mink showing typical paralysis of thiamine deficiency.

DISCUSSION

From the data presented it is evident that 120 μg of thiamine·HCl per 100 gm of ration supplies the thiamine requirement of the mink for growth and fur production. Increasing the level of thiamine·HCl in the mink's diet from 120 μg to 150 μg per 100 gm of ration did not result in superior growth or fur quality. The thiamine requirement of the mink is similar to that of the chick as determined by Jukes and Heitman ('40). These workers found that the thiamine requirement of the chick for optimal growth was between 130 and 150 μg per 100 gm of ration.

SUMMARY

It has been determined that 120 μg of thiamine·HCl per 100 gm of ration supplies the thiamine requirement of the mink for growth and fur development.

LITERATURE CITED

Basset, C. F., J. K. Loosli and F. J. Wilke 1948 The vitamin A requirement for growth of foxes and minks as influenced by ascorbic acid and potatoes. J. Nutrition, *35*: 629.

Basset, C. F., L. E. Harris and F. J. Wilke 1951 Effect of various levels of calcium, phosphorus and vitamin D intake on bone growth. II. Minks. Ibid., *44*: 433.

Green, R. G., W. E. Carlson and C. A. Evans 1941 A deficiency disease of foxes produced by feeding fish. Ibid., *21*: 243.

Jukes, T. H., and H. J. Heitman 1940 Biological assay of thiamine with chicks. Ibid., *19*: 21.

Leoschke, W. L., R. J. Lalor and C. A. Elvehjem 1933 The vitamin B_{12} requirement of mink. Ibid., *49:* 541.

Leoschke, W. L., and C. A. Elvehjem 1959 The importance of arginine and methionine for the growth and fur development of mink on purified diets. Ibid., *69:* 147.

Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constitutents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

Schaefer, A. E., C. K. Whitehair and C. A. Elvehjem 1946 Purified rations and the importance of folic acid in mink nutrition. Proc. Soc. Exp. Biol. Med., 62: 169.

Riboflavin in the Nutrition of the Chinchilla[1]

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Comparatively little is known about the nutritional requirements of the chinchilla. The only study reported in the literature on the vitamin requirements of the chinchilla is that of King and Orcutt ('52). Due to a limited number of animals available for experimental study, they considered their report on the thiamine requirement of the chinchilla as tentative. Their work indicated that ascorbic acid is not a dietary requirement of the chinchilla.

The development of a purified diet capable of supporting normal growth of the chinchilla has only recently been attained by Larrivee and Elvehjem ('54). This achievement has greatly facilitated the study of the vitamin requirements of the chinchilla. The chinchilla, like the rabbit and the guinea pig, has a large cecum. It is possible that the intestinal and cecal bacterial flora may make a substantial contribution to the B vitamin requirements of the chinchilla. Studies by Olcese, Pearson and Schweigert ('48) have demonstrated that rabbits do not need a dietary source of riboflavin. Their work indicated significant synthesis of riboflavin in the rabbit. The present study was designed to study the role of riboflavin in the nutrition of the chinchilla. The rate of growth and the urinary and fecal excretion of riboflavin were determined with chinchillas on a purified diet low in riboflavin.

EXPERIMENTAL AND RESULTS

The composition of the purified basal diet used in this study was similar to that used by Larrivee and Elvehjem ('54) and had the following composition: sucrose 39, casein[3] 30, cottonseed oil 4, salts IV (Phillips and Hart, '35) 4, potassium acetate 2.5, magnesium oxide 0.5 and cellulose[4] 20%. Each 100 gm of ration was supplemented with 1.0 mg thiamine·HCl, 1.0 mg pyridoxine·HCl, 1.4 mg riboflavin, 3.0 mg Ca pantothenate, 10 mg nicotinic acid, 300 mg choline·HCl, 200 mg *i*-inositol, 10 mg *p*-aminobenzoic acid, 0.2 mg 2 methyl 1-4 napthoquinone, 0.3 mg pteroylglutamic acid and 0.04 mg biotin. Haliver oil fortified with α-tocopherol acetate and vitamin D_3 was added so that 100 gm of ration contained 2,000 I.U. vitamin A, 200 I.U. vitamin D and 12 mg α-tocopherol acetate. This basal diet contained 14.4 μg of riboflavin per gm.

The low riboflavin diet consisted of the purified basal diet without supplemental riboflavin. This low-riboflavin diet contained 0.39 μg of riboflavin per gram. Forty male, weanling chinchillas were distributed on the basis of weight and genetic history into two groups of 20 animals each and fed the experimental diets. The average initial weights of the chinchillas fed the purified basal diet and the low-riboflavin diet were 255 and 243 gm respectively.

Growth of the chinchillas fed the purified basal and low riboflavin diets is shown in table 1. During the 5-month experimental period no symptoms of a riboflavin deficiency were observed in the group of chinchillas on the low riboflavin diet.

For the metabolism studies, 8 chinchillas were placed in individual metabolism cages and the urine and feces collected during two separate 24-hour periods. No special techniques were employed to prevent cop-

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[3] General Biochemicals, Inc.
[4] Solka Floc, Brown Company, Berlin, New Hampshire.

rophagy inasmuch as the chinchillas refused to eat when fitted with hard rubber collars designed to prevent coprophagy. The urine was collected in dark brown bottles containing 4 ml of 10% acetic acid. The feces were collected at the end of each 24-hour period. Food consumption was measured during each of the experimental periods. Urinary riboflavin was determined by the procedure of Ferreber ('40). Fecal riboflavin was liberated by autoclaving the samples with 0.1 N HCl. After autoclaving, the samples were cooled, adjusted to pH 4.5 with sodium acetate and filtered. The fecal samples were then analyzed for riboflavin by the method of Conner and Straub ('41). The results of the metabolism studies are summarized in table 2. It may be seen that the average riboflavin excretion of the chinchillas on the low-riboflavin diet is over 4 times the average intake of the vitamin.

TABLE 1

Growth of chinchillas fed purified basal and low-riboflavin diets

Diet	Gain, 5 months[1,2]
	gm
Purified basal	140 ± 10 (20)
Low-riboflavin	139 ± 9 (20)

[1] Mean and S.E.M.
[2] The number of chinchillas per group is indicated by the number within parentheses.

TABLE 2

Ingestion and excretion of riboflavin by chinchillas fed a low-riboflavin diet

Intake[1]	Excretion		
	Urine	Feces	Total
6.6 ± 0.8[2]	3.4 ± 0.5	24.5 ± 1.1	27.9 ± 1.2

[1] All values expressed as micrograms per 24-hours. Average of two different 24-hour collections.
[2] Mean and S.E.M.

DISCUSSION

From the data presented it is evident that dietary riboflavin is not required by the chinchilla. Growth of the chinchillas on the low-riboflavin diet containing 0.39 μg of riboflavin per gram is equal to that of the animals fed the purified basal diet containing 14.4 μg of riboflavin per gram. It is well known that diets this low in riboflavin will not support normal growth of the rat. Wagner et al. ('40) and Mannering et al. ('41) reported that the minimum daily requirement of riboflavin for the rat is between 18 and 30 μg. Assuming a daily feed consumption of 20 gm by the rat, the low-riboflavin diet would provide only 7.8 μg of riboflavin per day.

The data obtained on the excretion of riboflavin by the chinchilla clearly demonstrate that this vitamin is being synthesized by the chinchilla. The animals fed the low-riboflavin diet ingested an average of 6.6 μg of riboflavin daily and excreted an average of 27.9 μg. This represents a 4-fold increase in the amount excreted as compared to the amount ingested. The data on the growth and excretion of riboflavin of chinchillas fed low riboflavin diets is similar to that obtained by Olcese, Pearson and Schweigert ('48) in studies with rabbits.

SUMMARY

1. Riboflavin is not a dietary requirement for the chinchilla. A diet containing as little as 0.39 μg of riboflavin per gram produced normal growth in chinchillas. No symptoms of a vitamin deficiency were noted in animals fed the low-riboflavin diet for 5 months.

2. Metabolism studies with chinchillas fed the low riboflavin diet indicated a daily excretion of 27.9 μg corresponding to a daily intake of 6.6 μg of riboflavin per day.

LITERATURE CITED

Conner, R. T., and G. J. Straub 1941 Combined determination of riboflavin and thiamine in food products. Ind. Eng. Chem., Anal. Ed., *13:* 385.

Ferreber, J. N. 1940 The urinary excretion of riboflavin. Fluorometric method for determination. J. Clin. Invest., *19:* 251.

King, K. W., and F. S. Orcutt 1952 Nutritional studies of the chinchilla with special reference to ascorbic acid and thiamine. J. Nutrition, *48:* 31.

Larrivee, G. P., and C. A. Elvehjem 1954 Studies on the nutritional requirements of chinchillas. Ibid., 52: 427.

Mannering, G. J., M. A. Lipton and C. A. Elvehjem 1941 Relation of dietary fat to riboflavin requirement of growing rats. Proc. Soc. Exp. Biol. Med., *46:* 100.

Olcese, O., P. B. Pearson and B. S. Schweigert 1948 The synthesis of certain B vitamins by the rabbit. J. Nutrition, *35:* 577.

Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

Wagner, J. R., A. E. Axelrod, M. A. Lipton and C. A. Elvehjem 1940 A rat assay method for the determination of riboflavin. Ibid., *136:* 357.

Lysine and Threonine Supplementation of Rice

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Supplementation of a protein with its first limiting essential amino acid is highly beneficial if it brings the total amount of this amino acid present in the protein and available to the organism into balance with the second limiting amino acid according to body requirements (Rosenberg, '57). The validity of this concept has been amply demonstrated with a number of different proteins, e.g., wheat (Rosenberg and Rohdenburg, '52), rice (Rosenberg and Culik, '57), and corn (Rosenberg, '59) and with poultry diets of different protein quality (e.g., Rosenberg et al., '55; Baldini and Rosenberg, '55).

When proper balance has been reached between the first two limiting amino acids further improvement in protein quality should be attainable if both amino acids are added simultaneously and in the proportion of the body's requirements. It should be possible to add this amino acid mixture in graded amounts and to obtain increasing growth responses until a third amino acid, or other nutrient, becomes limiting. This concept has to the authors' knowledge not been tested experimentally. A study was undertaken, therefore, to check this theory using rice as the test protein and the growth of the male weanling rat as the biological criterion. The results of this study, reported here, support in general the above concept for supplementation of proteins with the first two limiting essential amino acids.

EXPERIMENTAL PROCEDURE

All experiments were carried out according to the previously described general plan (Rosenberg and Culik, '57). The composition of the basal diets is shown in table 1. The rice as well as the diets were repeatedly analyzed for their lysine and threonine content. The values according to microbiological assay were in agreement with those determined in our laboratories by the Moore-Stein chromatographic procedure and with the analysis published by Kik ('56). For the basal rice diets average values of 0.24% L-lysine and 0.23% L-threonine were obtained.

TABLE 1
Composition of diets

Ingredient	Diet A amount	Diet B amount
	%	%
Ground, precooked rice	91	90
Diammonium citrate	—	1
Vitamin mixture[1]	1.5	1.5
Salt mixture[1]	3.0	3.0
Cod liver oil	1.5	1.5
Crude soybean oil	3.0	3.0
Total	100.00	100.00

[1] The composition of the vitamin and the salt mixtures is described in Rosenberg and Culik ('57).

Diet A was used in most of the experiments. Supplementation of this diet with amino acids[1] was carried out by replacing rice. Diet B was used in the first three experiments and in the last 5 groups of experiment 11. When amino acids were added to diet B the amount of diammonium citrate was correspondingly reduced.

Weanling male animals were used from our colony of hooded rats. They were kept for a 5-week experimental period in individual cages with raised screen bottoms. Food and water were supplied ad libitum and individual weekly records were kept of weight gains and the amount of food consumed. Six animals were usually assigned to each treatment. In experiment 9 there were 10 animals and, in experiment 12, 25 litter mates per group. The diets and results of the 14 experiments are shown in tables 2, 3, and 4.

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[1] L-lysine·HCl (du Pont) containing 95% L-lysine·HCl and 5% D-lysine·HCl.

RESULTS AND DISCUSSION

In order to interpret the animals' responses from the first 11 experiments, involving 14 different levels of lysine in various combinations with 7 different levels of threonine, the data for gain were plotted on a three-dimensional graph. The amounts of total L-lysine and total L-threonine in the experimental diets were indicated on the ordinate and abscissa. The experimentally determined values for gain were plotted as the third dimension. The graph obtained resembled a mountain ridge, but there was considerable scatter of the individual points around a smooth ridge. To develop a logical explanation for this mountain ridge mathematical models were derived (see appendix) and were tested for agreement with the data by statistical methods. Constants were obtained from the experimental data by regression analysis. Equation 5 (appendix)

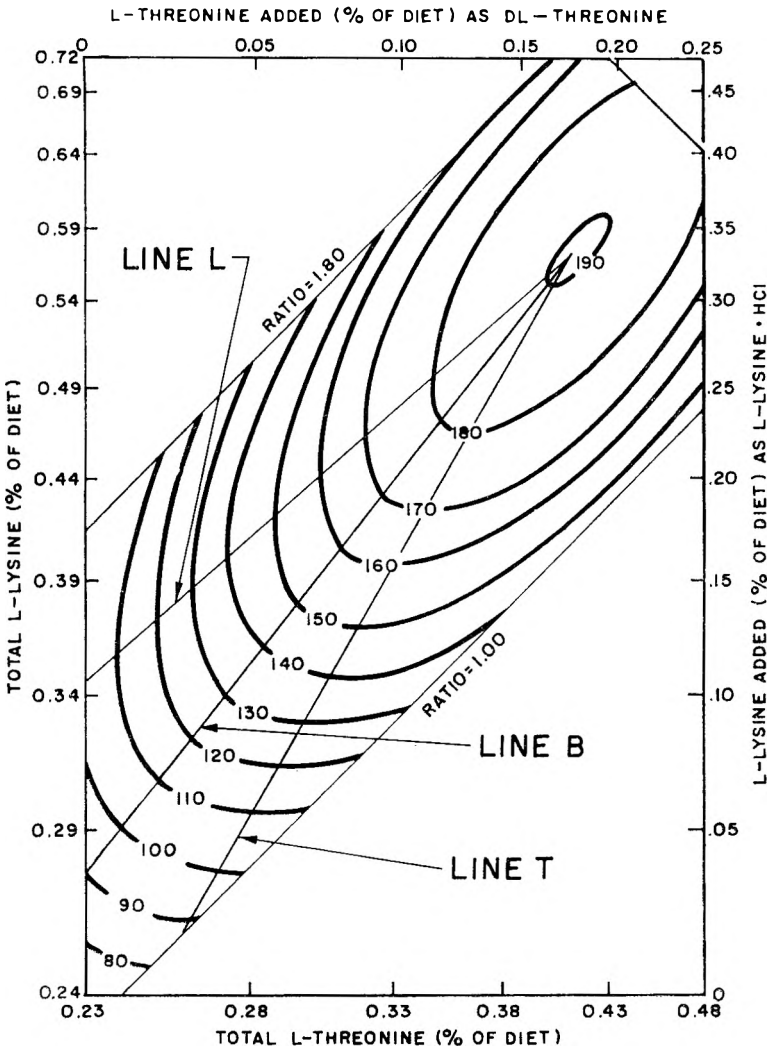

Fig. 1 Weight gain (grams) contours for male rats fed 5 weeks on rice diets supplemented with lysine and threonine. Based upon full-quadratic model, equation 5 in appendix. Line L, "line of optimum response" when threonine is constant and lysine is varied. Line T, "line of optimum response" when lysine is constant and threonine is varied. Line B is the "line of proper balance."

is in agreement with the data and is the best of the models.

Figure 1 shows the predicted average weight gains and figure 2 the predicted average efficiencies of feed utilization based on this mathematical model. These figures are contour maps of the expected responses due to supplementation of the basal rice diet with different amounts of the first two limiting amino acids. Changes in the weights gained and feed/gain correspond to changes in altitude on a geographical contour map. The total percentages of L-lysine and L-threonine are plotted on logarithmic scales because of the type of the equation. All ratios form positively sloped 45° lines with the amino acid axes.

Optimum gain is predicted to occur at about 0.57% of L-lysine and 0.41% of L-threonine, while optimum efficiency of food utilization is expected to occur at 0.60% of L-lysine and 0.43% of L-threonine. These points of best performance are not sharply delineated from other, nearby points. In the region of maximum performance relatively substantial changes in amino acid supplementation will bring about only small changes in the responses.

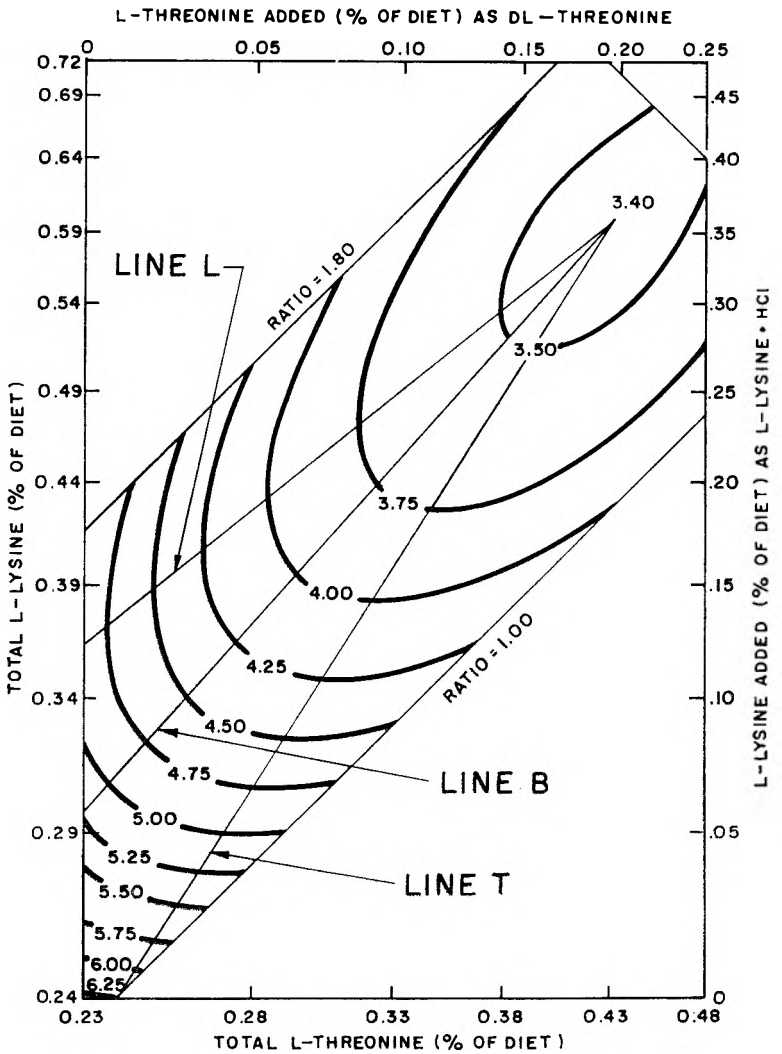

Fig. 2 Feed/gain contours for male rats fed 5 weeks on rice diets supplemented with lysine and threonine. Based upon full quadratic model, equation 6.

This is supported by the results of a special experiment designed to test the prediction of the mathematical model for the point of optimum performance. The data of experiment 12, table 2, show a slight advantage for the diet supplemented according to the prediction over a similar diet supplemented with somewhat larger amounts of lysine and threonine (to give best performance according to another model, see appendix).

The ratio of total lysine to total threonine in the diet at optimum response is 1.4 to 1 by weight. This is different from the ratio of 1.66:1 or 2.0:1 suggested by Rose ('37) and Rose et al. ('49) for the growing rat, but agrees with the ratio needed for repletion of the protein-depleted rat (Steffee et al., '50). However, in a study on the supplementation of bread protein, to be published separately, a ratio of L-lysine to L-threonine similar to that found by Rose was observed.

The numerical values for the total amount of each of the two amino acids at the point of optimum performance depend, of course, upon the amount present in the unsupplemented rice diet. If there is a difference between the analytical values and the amounts available to the growing rat, there should be a shift of the balance ratio with increasing supplementation. Such a shift was actually observed. The ratio rises from 1.2 for weight gain and 1.3 for feed/gain when no threonine is added to 1.4 at the point of optimum performance. This shift was significant at the 95% level for growth but not for feed efficiency.

Figures 1 and 2 illustrate that at suboptimal levels of lysine and threonine the same rate of gain and feed efficiency can be obtained by an infinite number of different combinations of the two limiting amino acids. Of all these combinations, the one which requires the smallest product of the two amino acids represents the *proper balance*. The *line of proper balance* connects the point of optimum performance with the points which give best response for a given product.

If the first two limiting amino acids of a protein are fully available, the line of proper balance should coincide with a ratio line. When drawn in arithmetic scales, the line of proper balance will then connect the point of optimum performance with the origin of the system (0% lysine, 0% threonine). When the amount of rice protein in the diet is increased or decreased, the ratio of lysine to threonine at the point of maximum attainable performance should follow the line of proper balance. This, however, was not tested experimentally.

The procedure used here to arrive at the proper balance between the first two limiting amino acids should, of course, be applicable to all proteins. It is conceivable that there is a specific balance characteristic for each protein, as the absolute amount of any one essential amino acid required for best balance may be affected by the amount of all the other amino acids in the test protein. In principle, the ratios for the essential amino acids determined according to this procedure should be considerably more accurate than the ratios found by previously used methods.

It was surprising to find that, as long as one of the two limiting amino acids is supplied in a suboptimal amount, the addition of a small amount of the other amino acid beyond the quantity required for "proper balance" improves slightly growth and efficiency of food utilization. Best performance under these conditions is obtained at levels of the second amino acid indicated in figures 1 and 2 as the *optimum response lines*, one for each set of conditions. The two optimum response lines are thus found on either side of the line of proper balance, fanning out from the point of maximum performance. Mass action is a possible explanation of this phenomenon. According to this principle, one amino acid when present in slight excess over a second amino acid would permit a more complete utilization of the second amino acid, thus allowing for increased protein synthesis and for increased overall growth. Of course, if the excess is large, the amino acid could function as a diluent opposed to the beneficial effect of mass action and actually retard the rate of protein synthesis.

The concept that mass action governs protein synthesis is in accord with the experience gained from studies with enzymes. The total amounts of L-lysine and L-threonine found in our experiments to be

TABLE 2

Effect of supplementation of rice diet with lysine and threonine

Supplementation		Responses	
L-lysine·HCl	DL-threonine	Average wt. gain	Feed/gain
%	%	gm	
	Experiment 1		
—	—	60*	6.95
—	0.05	51*	7.66
—	0.10	56*	6.74
—	0.20	56*	7.28
—	0.30	54*	7.10
	Experiment 2		
—	—	61*	6.37
0.05	—	88*	5.34
0.05	0.05	82*	5.32
0.05	0.10	88*	5.40
0.05	0.20	96*	5.14
0.05	0.30	91*	5.38
	Experiment 3		
—	—	57*	6.63
0.1	—	78*	5.14
0.1	0.05	117*	4.49
0.1	0.10	112*	4.41
0.1	0.20	138*	4.15
0.1	0.30	114*	4.47
0.2	—	81*	4.95
0.2	0.05	157*	3.87
0.2	0.10	135*	4.30
0.2	0.20	161*	3.84
0.2	0.30	151*	3.79
	Experiment 4		
—	—	72	5.99
—	0.05	70	6.22
—	0.10	80	5.70
—	0.20	81	5.73
—	0.30	80	5.84
0.025	0.05	87	5.71
0.05	0.05	98	5.16
0.10	0.05	135	4.45
0.15	0.05	127	4.34
0.20	0.05	126	4.35
0.25	0.05	122	4.32
0.10	—	106	4.79
	Experiment 5		
—	—	82	6.21
—	0.1	71	6.67
0.05	0.1	106	5.25
0.10	0.1	125	4.88
0.15	0.1	152	4.28
0.20	0.1	155	4.08
0.25	0.1	166	3.88
0.30	0.1	159	4.04
	Experiment 6		
—	—	61	6.94
—	0.2	46	8.39
0.10	0.2	109	4.85
0.15	0.2	142	4.37
0.20	0.2	145	4.13
0.25	0.2	146	4.03
0.30	0.2	158	3.74
0.35	0.2	189	3.58
	Experiment 7		
—	—	66	6.54
—	0.1	67	6.38
0.05	0.1	84	5.70
0.10	0.1	114	4.79
0.15	0.1	108	4.85
0.20	0.1	136	4.23
0.25	0.1	152	3.96
0.30	0.1	131	4.33
—	0.2	64	6.63
0.10	0.2	113	4.76
0.20	0.2	146	4.04
0.30	0.2	154	3.83
0.35	0.2	150	4.03
0.40	0.2	156	3.77
0.45	0.2	160	3.66
	Experiment 8		
—	—	80	6.05
—	0.3	78	6.20
0.2	0.3	180	3.71
0.3	0.3	179	3.63
0.4	0.3	171	3.72
0.5	0.3	187	3.54
0.6	0.3	190	3.50
—	0.4	91	5.65
0.3	0.4	146	4.00
0.4	0.4	184	3.61
0.5	0.4	178	3.62
0.6	0.4	191	3.43
0.7	0.4	161	3.81
0.35	0.2	198	3.49
0.6	0.4	191	3.51
	Experiment 9		
0.10	0.05	114	4.53
0.25	0.10	137	4.07
0.35	0.20	159	3.76
	Experiment 10		
—	—	70	6.45
0.60	0.40	167	3.78
	Experiment 11		
—	—	79	5.61
0.10	—	94	4.98
0.10	0.05	111	4.20
0.10	0.10	127	4.37
0.10	0.20	130	4.30
0.10	0.30	132	4.17
0.35	—	73	5.46
0.35	0.10	164	3.67
0.35	0.20	173	3.54
0.35	0.30	166	3.51
0.35	0.40	177	3.48
0.50	—	62	5.96
0.50	0.20	193	3.32
0.50	0.30	163	3.60
0.50	0.40	174	3.49
0.50	0.50	198	3.28
0.10	—	74*	5.78
0.10	0.05	93*	4.96
0.10	0.10	105*	4.79
0.10	0.20	110*	4.69
0.10	0.30	104*	4.86
	Experiment 12		
0.475	0.50	157	3.75
0.425	0.38	166	3.72

* Diammonium citrate added.

needed for optimum performance should then be subject to the same forces and are, therefore, probably larger than required for proper balance with the next limiting amino acid.

It is realized, of course, that the improved performance from supplementation with one amino acid beyond the amount required for proper balance with the other amino acid was noted while the diet contained a suboptimal level of total protein. Nevertheless, this effect is not a non-specific response to increased nitrogen needed for protein anabolism. Addition of extra nitrogen in the form of diammonium citrate actually produced a detrimental effect.

In view of the new phenomena observed, the question arises whether or not other proteins and other amino acids will show a similar balance behavior. To answer this question the supplementation of corn and of wheat diets with the first two limiting essential amino acids was investigated. The same type of phenomenon was indeed observed. These findings, details of which will be published separately, contribute to the confidence of the results obtained with rice.

We believe these results demonstrate that threonine is the second limiting amino acid in the rice protein. Pecora and Hundley ('51) have shown earlier that the combination of lysine and threonine was unique in eliciting a substantial growth response in rats fed a rice diet. A reinvestigation under the conditions of our experiments, however, would not necessarily be expected to yield the same information since the former workers had tested combinations of lysine with all other essential amino acids only at the levels of the rat's requirements as suggested by Rose ('37). Waddell ('58), using the amino acid analyses of rice protein published by Pecora and Hundley ('51) estimated that histidine

TABLE 3

Effect of histidine addition to lysine-supplemented rice diet
(Five-week growth data of 6 male and 6 female rats)

Supplementation	Average weight gain	Feed/gain
	gm	
None	69	6.14
0.2% L-lysine·HCl	80	5.23
0.2% L-lysine·HCl + 0.05% histidine·HCl	81	5.44
0.2% L-lysine·HCl + 0.10% histidine·HCl	67	5.97
0.2% L-lysine·HCl + 0.15% histidine·HCl	79	5.54

TABLE 4

Effect of amino acid supplementation of rice diet
(Five-week growth data of 6 male rats per group)

Group	Supplementation	Average weight gain	Feed/gain
		gm	
1	None	70	6.45
2	0.6% L-lysine·HCl + 0.4% DL-threonine	167	3.78
3	As in 2, + 0.1% DL-methionine + 0.22% DL-isoleucine + 0.05% DL-tryptophan	191	3.31
4	As in 3, + 0.03% L-valine	193	3.26
5	0.7% L-lysine·HCl + 0.4% DL-threonine + 0.3% DL-methionine + 0.22% DL-isoleucine + 0.12% DL-tryptophan + 0.10% DL-valine + 0.30% DL-phenylalanine + 0.23% L-leucine + 0.31% L-histidine·HCl	215	3.15
6	Stock diet	238	2.75

and not threonine should be the second limiting amino acid. We have carried out a special experiment, therefore, in which successively increasing amounts of histidine were added to the rice diet, together with 0.2% of L-lysine·HCl. As seen from table 3, no growth responses were obtained, thus ruling out histidine as the second limiting amino acid. This is also in agreement with the more recent amino acid analysis of rice by Kik ('56), which was confirmed in our laboratories.

Having established lysine and threonine as the first and second limiting amino acids in rice protein for the growth of the weanling rat, it was of interest to determine if the addition of the next limiting amino acids to the diet supplemented with lysine and threonine would elicit a growth response. Table 4 shows the results of an exploratory experiment in which methionine, isoleucine, and tryptophan were added simultaneously. As a group they represent the third limiting amino acids according to present knowledge of the growing rat's amino acid requirements. A substantial improvement in performance was obtained. The further addition of valine did not result in a significant response. When, finally, phenylalanine, leucine, and histidine were added to all others, further growth improvement resulted which brought the response up to 90% of that obtained with our stock diet. It is probably fair to assume that all major amino acid deficiencies were satisfied. The remaining 10% additional growth could probably be obtained if it were possible to add the various amino acids in such amounts that in the final mixture the ideal ratios resulted, and to adjust the energy content of the diet to the amount of this ideal protein (Rosenberg, '57).

SUMMARY

Precooked rice diets, supplemented with graded amounts of lysine and threonine, the first two limiting essential amino acids, were fed to over 800 weanling male rats. Fourteen different levels of lysine were tested in various combinations with 7 different levels of threonine. Equations expressing the animal's growth and efficiency of feed utilization were developed and verified by regression analysis using the Univac. According to these calculations best performance occurs when 0.34% of lysine (0.425% L-lysine·HCl) and 0.18% of threonine (0.36% DL-threonine) are added to this rice diet. On the basis of the microbiologically determined lysine and threonine content of the unsupplemented rice diet, the diet supplemented for optimum performance contains 1.4 times as much L-lysine as L-threonine.

When the amount of one amino acid is held constant at a suboptimal level and the other varied, a slightly greater amount of the second is required than expected from the above ratio to give optimal performance. Mass action is a possible explanation of this phenomenon.

The experimental results support the principle that multiple essential amino acid supplementation of a protein should be carried out in such a manner that, according to the needs of the organism, the supplemented amino acids are present in the proper ratio to each other and in balance with the next limiting amino acid or nutrient in the diet.

ACKNOWLEDGMENTS

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APPENDIX

Mathematical evaluation of experimental data

In the experiments described, growth and efficiency of food utilization of the animals consuming the basal diet are limited by the amounts of the two essential amino acids lysine and threonine. Thus the equilibrium of the overall biochemical reaction which limits weight gain, G, involves l moles of lysine (concentration of L) and t moles of threonine (concentration of T) while all other diet ingredients are of fixed concentration and are included with the equilibrium constant as k.

$$G = kL^l T^t \qquad (1)$$

For statistical calculations to evaluate the

unknown constants the logarithmic form is more convenient.

$$\ln G = \ln k + l \ln L + t \ln T \qquad (2)$$

This equation, which is an expression of mass action, does describe the general pattern of results obtained, but it cannot account for the observed detrimental effect of excesses of lysine. Therefore, quadratic terms were added to the basic model to give a second degree Taylor Series.

$$\ln G = \ln k + l \ln L + t \ln T + g(\ln L)^2 + d(\ln T)^2 + f \ln L \ln T \qquad (3)$$

where g, d, and f are additional constants.

Because of the importance attached to the ratio of essential amino acids, equation 3 is rewritten in terms of the ratio and product of the two amino acids:

$$\ln G = \ln k + r \ln \frac{L}{T} + p \ln LT + m(\ln \frac{L}{T})^2 + n(\ln LT)^2 + q \ln \frac{L}{T} \ln LT \qquad (4)$$

where k, r, p, m, n, and q are constants to be evaluated from the experimental data. Actually equations 3 and 4 are equivalent and the constants can be related. The mathematical model developed here must be considered an empirical one although based on a physiological rationale because we obviously are not directly observing the isolated reactions described.

Major interest in this two amino acid system has been centered on regions of favorable response with minimum supplementation. Examination of the experimental data revealed no favorable trends outside the limits of L-lysine to L-threonine ratio between 1.0 and 1.8:1, based on total amino acid content of the diet. Therefore, the 80 groups of animals from experiments 1 to 11, table 2, and the 15 groups from the earlier work (Rosenberg and Culik, '57), within these limits were used for calculation.

Statistical evaluation indicated that the data from these experiments could be combined without undue concern about the differences among experiments. The coefficients of variation for group averages among experiments and within experiments were found to be 11.3% and 11.0% for group average weight gain and 5.7% and 5.3%, respectively, for efficiency of feed utilization.

The constants for equation 4 were obtained from the experimental data by regression analysis using the Univac computer. The evaluated equation for weight gain with a ratio of L-lysine to L-threonine between 1.0 and 1.8 follows:

$$\ln G = 3.646 - 0.121D - 1.600(\ln LT) + 2.674(\ln \frac{L}{T}) - 0.478(\ln LT)^2 - 2.662(\ln \frac{L}{T})^2 + 0.630(\ln LT)(\ln \frac{L}{T}) \qquad (5)$$

The corresponding equation for feed efficiency follows:

$$-\ln \frac{G}{F} = \ln \frac{F}{G} = 1.909 + 0.034D + 0.712(\ln LT) - 1.154(\ln \frac{L}{T}) + 0.240(\ln LT)^2 + 1.387(\ln \frac{L}{T})^2 - 1.80(\ln LT)(\ln \frac{L}{T}) \qquad (6)$$

where F is the feed consumed. The term D allows for the effect of diammonium citrate on the responses of the animals. If diammonium citrate was added, D is one; otherwise, it is zero. The presence of 1% of this additive in the feed decreased weight gain 12% and gain/feed 4%.

These full-quadratic equations are adequate descriptions of the experimental data for both responses. The "lack-of-fit" does not differ significantly from that expected due to biological and other variation for either response (see Regression Analysis, table 5).

These equations allow for a balance ratio which changes with the actual amounts of amino acids in the diet. Without the last term the equation forces a fit of the single best balance ratio as follows:

$$\ln G = 4.658 - 0.134D - 0.846(\ln LT) + 0.985(\ln \frac{L}{T}) - 0.340(\ln LT)^2 - 2.112(\ln \frac{L}{T})^2 \qquad (7)$$

$$-\ln \frac{G}{F} = \ln \frac{F}{G} = 1.620 + 0.038D + 0.497(\ln LT) - 0.672(\ln \frac{L}{T}) + 0.201(\ln LT)^2 + 1.229(\ln \frac{L}{T}) \qquad (8)$$

The importance of the last term in both equations 5 and 6 can be tested by statistical methods. It was found that the contribution of this term to the weight gain equation is not significant at the 99% level but is at the 95% level. For feed efficiency

this term is not significant at any reasonable level (table 5). Therefore, there is some evidence for a varying ratio of proper balance between the two essential amino acids. Small deviations from a constant balance ratio could be due to error in the analysis of the basal feed and to a more limited availability of one amino acid over the other from the rice protein. The "ratio" model, equations 7 and 8, is considered to be a useful description of the responses because it is in accord with present nutritional concepts, but the "full-quadratic" model should be slightly more accurate. The contour map of the predicted changes in weight gain according to the ratio model within the limits of the experiments analyzed and without diammonium citrate in the feed is shown in figure 3.

Examination of these equations reveals a number of specific results. *Optimum performance* of the animals is obtained when some specific amount of lysine and of threonine is added to the basal rice diet. To calculate this point, the partial derivatives of the response function are taken with respect to each of the two variables (the ratio, L/T, and the product, LT) and

TABLE 5

Regression analysis table: ratio and full-quadratic equations

Source of variation	Degrees of freedom	Sum of squares: Around the mean	Sum of squares: %	Mean square	F ratio	Critical values: F 0.99	Critical values: F 0.95
Weight gain							
Total	94	11.08944	100.00				
Regression[1]	5	9.89622	89.24	1.97924	157.08	3.42	
	1	0.08438	0.76	0.08438	6.69	7.19	4.04
Residual	88	1.10894	10.00	0.01260			
Lack of fit	39	0.57872		0.01484	1.37		1.65
Error (replicates)	49	0.53022		0.01082			
Feed/gain							
Total	94	3.92954	100.00				
Regression[1]	5	3.57627	91.01	0.71525	181.53	3.42	
	1	0.00707	0.18	0.00707	1.79		4.04
Residual	88	0.34619	8.81	0.00394			
Lack of fit	39	0.19369		0.00496	1.59		1.65
Error (replicates)	49	0.15250		0.00311			

[1] The 5 degrees of freedom are for the ratio equation and the additional one degree of freedom completes the full-quadratic equation.

TABLE 6

Regression analysis table: straight-line contour equation

Source of variation	Degrees of freedom	Sum of squares: Around the mean	Sum of squares: %	Mean square	F ratio	Critical values: F 0.99	Critical values: F 0.95
Weight gain							
Total	94	11.08944	100.00				
Regression	4	9.84820	88.81				
Residual	90	1.24124	11.19	0.01379			
Lack of fit	41	0.71102		0.01734	1.60	2.02	1.64
Error (replicates)	49	0.53022		0.01082			

each is set equal to zero. Solution of the two resulting simultaneous equations gives the coordinates of the point of maximum response. Thus, for example, from equation 7:

$$\left[\frac{\partial \ln G}{\partial \ln LT}\right]_{\ln \frac{L}{T} = \text{constant}} = -0.846 - 0.680 \ln LT = 0 \quad (9)$$

$$\left[\frac{\partial \ln G}{\partial \ln \frac{L}{T}}\right]_{\ln LT = \text{constant}} = 0.985 - 4.224 \ln \frac{L}{T} = 0 \quad (10)$$

According to these equations maximum gain in weight occurs with a total L-lysine content of 0.60% and L-threonine content of 0.48%. For feed utilization equation 8 indicates best efficiency to occur at 0.63% L-lysine and 0.48% L-threonine. On the basis of the full-quadratic formulae 5 and 6, optimum gain occurs at 0.57% of L-lysine and 0.41% of L-threonine, while optimum efficiency of feed utilization occurs at 0.60% of L-lysine and 0.43% of L-threonine. The points of best performance do not coincide for the two equations, especially not in the amount of threonine.

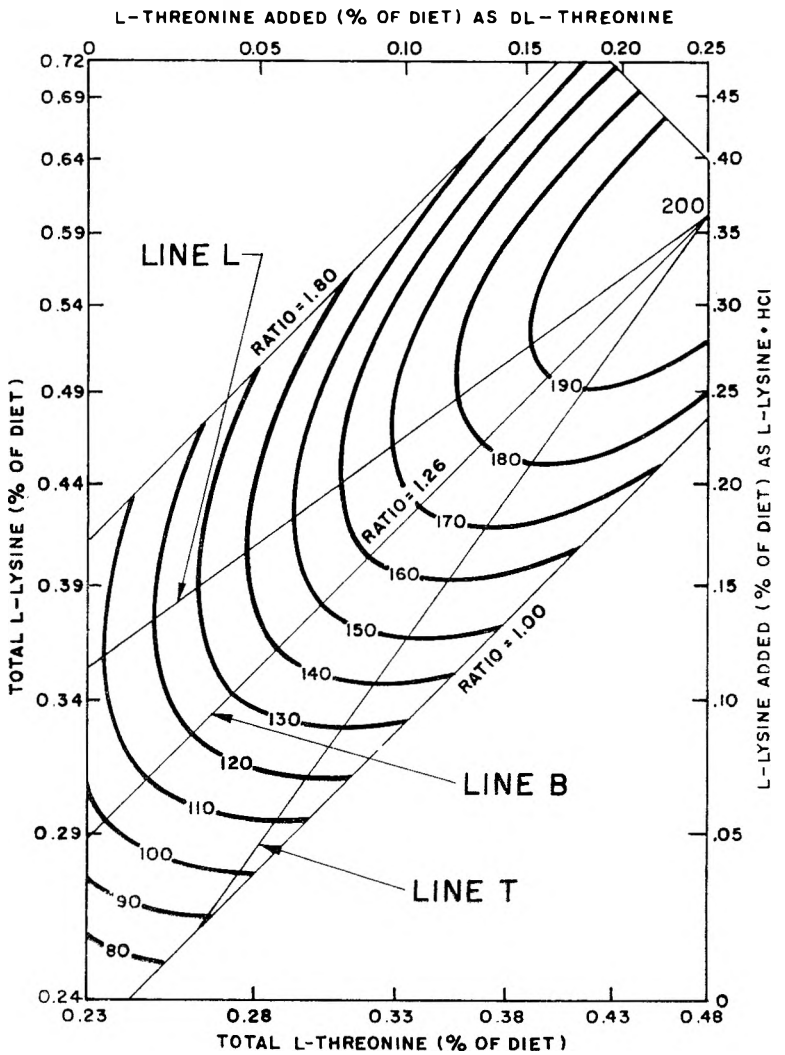

Fig. 3 Weight gain (grams) contours for male rats fed 5 weeks on rice diets supplemented with lysine and threonine. Based upon ratio model, equation 7.

A special experiment carried out to compare the two diets (experiment 12, table 2) indicated a numerical advantage for the results from the full-quadratic model which, however, was not statistically significant. Less weight was gained in this experiment because considerably heavier (older) rats were used to obtain the 25 litter mates. A line of *proper balance* between the two amino acids, as previously defined is equation 10.

Optimum response lines when one of the amino acids is held constant and the other varied are obtained when the partial derivative of the response function with respect to the amino acid varied is set equal to zero. For example, the partial derivative of equation 7 with respect to lysine holding threonine constant gives the following line as a locus of possible maximum weight gain when only lysine is varied:

$$\left[\frac{\partial \ln G}{\partial L}\right]_{T = \text{constant}} = 0.139 - 4.904 \ln L + 3.544 \ln T = 0 \qquad (11)$$

Thus, adding only lysine to the basal diet, maximum weight gain occurs at 0.36% of L-lysine and minimum feed/gain

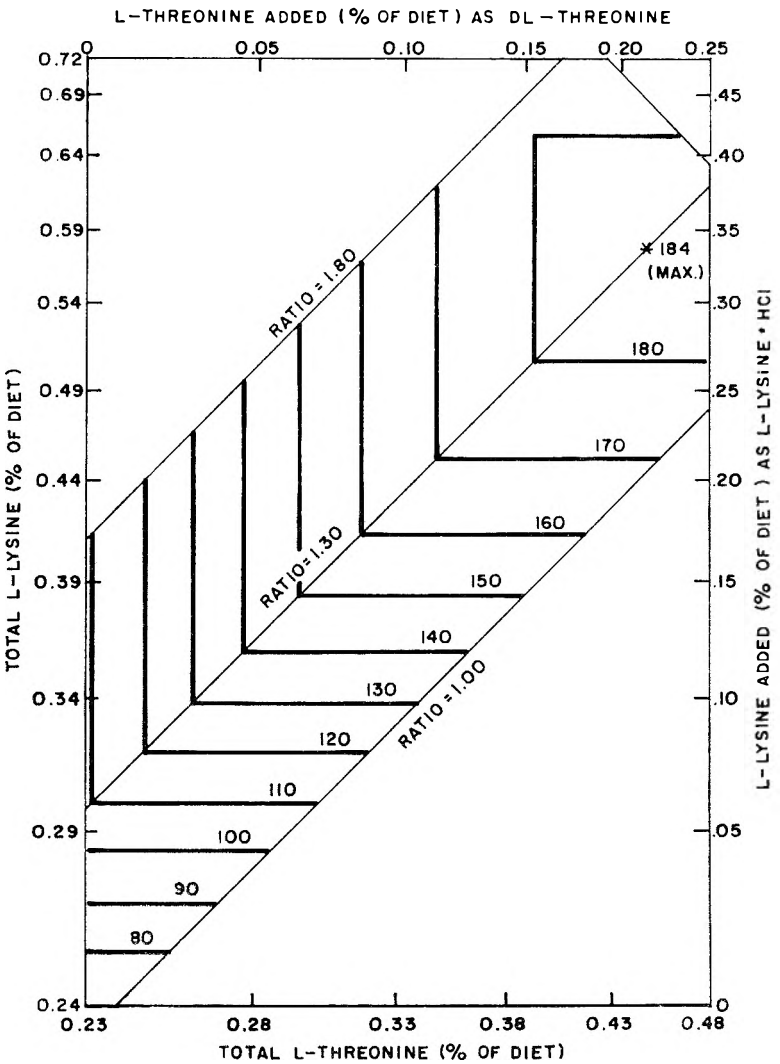

Fig. 4 Weight gain (grams) contours for male rats fed 5 weeks on rice diets supplemented with lysine and threonine. Based upon straight line contour model, equation 12.

at 0.37% of L-lysine. The corresponding ratios are 1.55 and 1.60. It is interesting to note that previously reported balance ratios for L-lysine to L-threonine approximate 1.6. This value might have been determined by varying only lysine. Such a procedure locates the maximum for a fixed amount of one amino acid. However, this cannot be considered proper balance because fixing the other acid gives a different line of maxima. The amount of either acid to add to obtain maximum response can be readily estimated from the equations or graphs.

Straight line contour equation. The idea for this equation was considered initially when it was assumed that the same line of maximal performance should be attained varying either amino acid. Study of the contour maps (figs. 1 to 3) shows that this can occur only if the contours meet at the balance line at an angle of 90° or less. The least drastic case occurs when straight lines meet at right angles. Figure 4 shows the best contours of this type calculated from these data using the equation:

$$\ln G = 4.841 - 0.136D - 1.358Z - 1.233Z^2 \quad (12)$$

and

$$-\ln\frac{G}{F} = \ln\frac{F}{G}$$

$$= 1.487 + 0.039D + 0.844Z + 0.768Z^2 \quad (13)$$

where for

$\frac{L}{T}$ greater than R (balance ratio), $Z = \ln T + \ln R$

$\frac{L}{T}$ less than R, $Z = \ln L$

This model cannot be evaluated directly by regression techniques but requires a prior selection of the balance ratio. R = 1.30 was used for the above evaluations. The best estimates of the constants actually require that the R be selected for best fit of the data. Such a lengthy calculation was not considered worthwhile as the previous calculations showed that the balance ratio was near 1.30. Comparison of this model with the full-quadratic shows that the latter is better on the basis of the statistical analysis (see tables 5 and 6).

The full-quadratic or ratio model also appears more acceptable in view of the general behavior of biological systems. A sharp discontinuity in contour lines such as required by the straight line model is not likely to occur except under very unusual conditions, for example when there is an abrupt discontinuity of a major factor affecting the organism. Thus, there is no evidence to support such a critical knife-edged ridge of proper amino acid balance.

LITERATURE CITED

Baldini, J. T., and H. R. Rosenberg 1955 The effect of productive energy level of the diet on the methionine requirement of the chick. Poultry Sci., *34:* 1301.

Kik, M. C. 1956 Nutritional improvement of rice. J. Am. Dietetic Assoc., *32:* 647.

Pecora, L. J., and J. M. Hundley 1951 Nutritional improvement of white polished rice by the addition of lysine and threonine. J. Nutrition, *44:* 101.

Rose, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, *86:* 298.

Rose, W. C., L. C. Smith, M. Womack and M. Shane 1949 The utilization of the nitrogen of ammonium salts, urea, and certain other compounds in the synthesis of non-essential amino acids *in vivo*. J. Biol. Chem., *181:* 307.

Rosenberg, H. R. 1957 Methionine and lysine supplementation of animal feeds. J. Agr. Food Chem., *5:* 694.

——— 1959 Supplementation of foods with amino acids. Ibid., *7:* 316.

Rosenberg, H. R., J. T. Baldini, M. L. Sunde, H. R. Bird and T. D. Runnels 1955 The concomitant use of fat and methionine in broiler diets. Poultry Sci., *34:* 1308.

Rosenberg, H. R., and R. Culik 1957 The improvement of the protein quality of white rice by lysine supplementation. J. Nutrition, *63:* 477.

Rosenberg, H. R., and E. L. Rohdenburg 1952 The fortification of bread with lysine. II. The nutritional value of fortified bread. Arch. Biochem. Biophys., *37:* 461.

Steffee, C. H., R. W. Wissler, E. M. Humphreys, E. P. Benditt, R. W. Woolridge and P. R. Cannon 1950 The determination of minimum daily essential amino acid requirements in protein-depleted adult male albino rats. J. Nutrition, *40:* 483.

Waddell, J. 1958 Supplementation of plant proteins with amino acids. In: Altschul, A. M., Processed Plant Proteins. Academic Press, N. Y., pp. 307–351.

Effect of Thiamine Deficiency and Thiamine Injection on Total Liver Lipids, Phospholipid, Plasmalogen and Cholesterol in the Rat[1]

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Certain relationships of thiamine deficiency to lipid metabolism have been studied by earlier investigators. McHenry and coworkers (McHenry, '35, '37; McHenry and Gavin, '38; Gavin and McHenry, '40) observed in the rat that as thiamine stores are exhausted in a double deficiency of choline and thiamine, liver lipid decreases markedly. Thus, production of a fatty liver in choline deficiency appears to be quite dependent on the presence of thiamine. Boxer and Stettin ('44) found in the rat that a thiamine deficiency produces a loss in liver lipid but attributed this effect mainly to depressed food intake. Arnold and Elvehjem ('39) reported that the inclusion of high levels of fat in the diet can cut the thiamine requirement to one-third that on a fat-free diet. Rubino et al. ('56) observed that, when rats were starved and then refed, there was a significantly greater deposition of total body fat when thiamine was included in the diet being refed. Engel and Phillips ('39) have shown that injection of thiamine into thiamine-deficient chicks produces a fatty metamorphosis in the parenchyma of the liver as well as increases in total glycogen and water content of the liver. In these studies phospholipid and protein remained unchanged. Thus it appears that thiamine may have a more directly significant role in lipid metabolism than simply to promote lipid deposition by increasing appetite or acting via pyruvate decarboxylase to produce lipid from carbohydrate. In view of the interrelationship between thiamine and choline reported by McHenry and coworkers, it is entirely possible that thiamine may be involved in fat mobilization and storage by an as yet unknown mechanism. At present, the authors do not believe that the entire question of the role of thiamine in lipid metabolism has been settled.

Of particular interest in the present studies has been a possible role that thiamine might play in maintaining liver plasmalogens. The plasmalogens comprise a group of phospholipids which give rise to long-chain aldehydes when hydrolyzed by acid (Feulgen and Voit, '24). It has been reported that the aldehydogenic group is attached to the glycerol moiety of the molecule either at the α- or β-carbon atom by a vinyl ether linkage (Rapport et al., '57; Marinetti et al., '58, '59). Recently, considerable interest in these cellular compounds has been aroused. During stress conditions they are considerably elevated (Hornykiewytsch and Geydl, '51; Yarbro and Anderson, '56, '57). They are also a part of a lipoprotein isolated from particulate succinic oxidase complex (Basford, '58; Joel et al., '59). Although the plasmalogens are widely distributed in nature, being found in both animals and plants, practically nothing is known of their function or metabolism. The metabolic source of the long-chain aldehydogenic component of the molecule is unknown. Thiamine is known to be involved in carbohydrate metabolism by acting in the decarboxylation of pyruvate. Green et al. ('42) showed that a thiamine-containing enzyme is capable of decarboxylating not only pyruvate but also α-ketoglutarate and α-ketobutyrate. By analogy, a possible mechanism by which the aldehyde-yield-

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ing portion of the plasmalogen molecule could arise is by decarboxylation of a long-chain α-keto acid. Thus in the present experiments the plasmalogen concentration of liver was followed, during the development of a thiamine deficiency, to observe whether the production of these compounds is influenced by thiamine.

EXPERIMENTAL

One hundred weanling male rats of the Sprague-Dawley strain were fed a complete stock ration until they reached 70 gm, then separated into two groups. Group I (26 rats) was fed a complete purified ration consisting of 20% of casein,[3] 0.3% of DL-methionine, 5% of corn oil, 4% of Salts IV (Hegsted et al., '41), 2% of a complete vitamin mix in sucrose as previously described (Williams and Elvehjem, '49), and sucrose to make 100%. They were given fat-soluble vitamins orally in cod-liver oil to furnish 6 I.U. of vitamin A, 0.06 I.U. of vitamin D, 0.04 mg of menadione, and 0.5 mg of α-tocopherol per rat per week. The second group (II), comprised of 74 rats, was fed the same ration except that thiamine was omitted from the vitamin mix. All rats were given water ad libitum.

At zero time 8 rats from group I were sacrificed by decapitation. Their livers were removed and weighed, and 4 gm of liver were homogenized in a Waring Blendor with 20 ml of 80% ethanol plus 60 ml of petroleum ether (b.p. of 30 to 60°C) for 4 minutes. The petroleum ether phase was separated and saved. The liver-ethanol phase was rehomogenized twice more with 60 ml of petroleum ether, and the petroleum ether extracts were pooled with the first petroleum ether extract. The petroleum ether was removed *in vacuo* at 50°C. The lipid residue was extracted with 50 ml of petroleum ether and then filtered. All extracts were analyzed for total lipid phosphorus by the method of Fiske and SubbaRow ('25) and plasmalogens by the method of Feulgen, Boguth and Andresen ('51). Total lipids were determined by evaporating the solvent from an aliquot of the extract in a weighed vessel and reweighing to find the weight of the lipid residue.

At 4- to 5-day intervals after placing the animals of group II on the thiamine-deficient ration, 6 rats were sacrificed, and phospholipid, total lipid, and plasmalogen estimated as above. Beginning with the 8th group of the thiamine-deficient rats to be sacrificed (starting on day 40), total cholesterol of the lipid extracts was determined by the method of Pearson et al. ('53).

The remainder of the animals receiving the complete ration (group I) were sacrificed in groups of 6 after 18, 44, and 55 days, respectively.

On day 49, the remaining rats fed the thiamine-deficient ration were injected intraperitoneally with 1 mg of thiamine in water. After two hours and 4 hours, two groups of three rats were sacrificed. After 48 hours, the last 4 animals in the group were sacrificed.

RESULTS

The results, presented graphically in figures 1 to 5, are reported both as micromoles of component measured per gram of fresh wet liver and as micromoles of component measured per 100 gm of body weight. The latter method of representation allows an adjustment of the lipid component to an equivalent body weight both for controls and thiamine-deficient rats. This is important since the body weights of the deficient group after 10 days was much less than that of the controls (fig. 1).

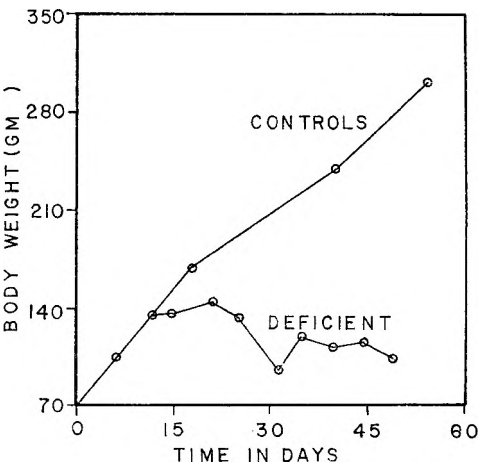

Fig. 1 Change in weight of rats fed the complete and the thiamine-deficient rations.

[3] Vitamin Test, General Biochemicals, Inc.

The total lipids in thiamine deficiency (fig. 2) began to decrease immediately after the start of the regimen. By the 31st day the total lipid reached a level that was only slightly higher than the phospholipids (fig. 3) indicating that the livers had been almost completely depleted of neutral lipid. When the rats were injected with thiamine (day 49), there was a sharp rise in total lipid to a high normal level after 48 hours. However, this change did not occur by 4 hours after thiamine injection, as indicated by the unreported results for animals injected and sacrificed after two and 4 hours. Over the 48-hour interval the liver weights increased 2.5-fold and changed from a deep red color and a dry texture to a light tan color and a very friable texture. The iodine numbers, run on samples of the lipid extracts from the thiamine-injected animals, averaged 84 as compared with 75 for the normal controls and 121 for the thiamine-deficient group sacrificed on day 49. The iodine number of the corn oil fed was 97.

The phospholipids (fig. 3) showed very little change throughout the experiment when calculated per gram of liver. However, when calculated per 100 gm of body weight the phospholipids were consistently lower than in the normal controls. When

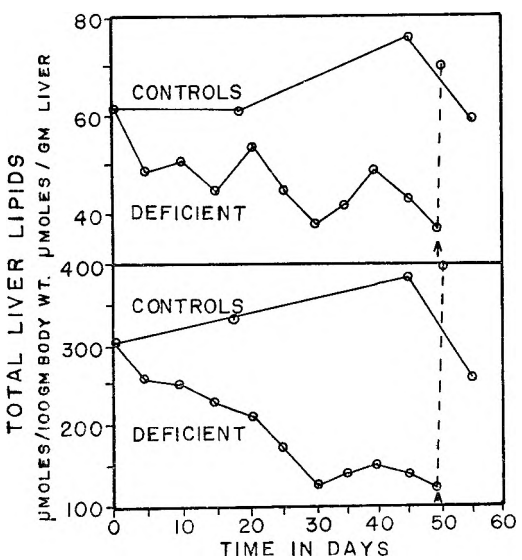

Fig. 2 Change in total liver lipids *versus* time, in rats fed the control and thiamine-deficient rations. One milligram of thiamine was injected into each deficient rat at the arrow.

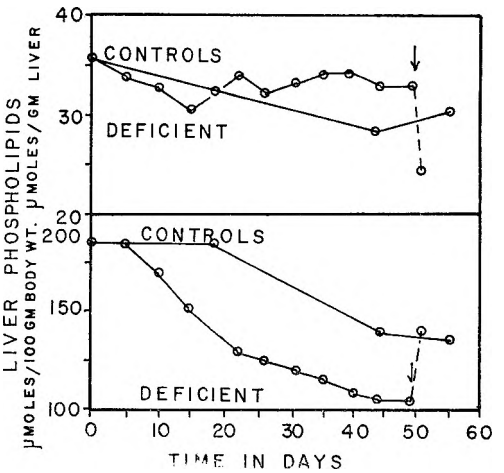

Fig. 3 Change in liver phospholipids *versus* time, in rats fed the control and thiamine-deficient rations. One milligram of thiamine was injected into each deficient rat at the arrow.

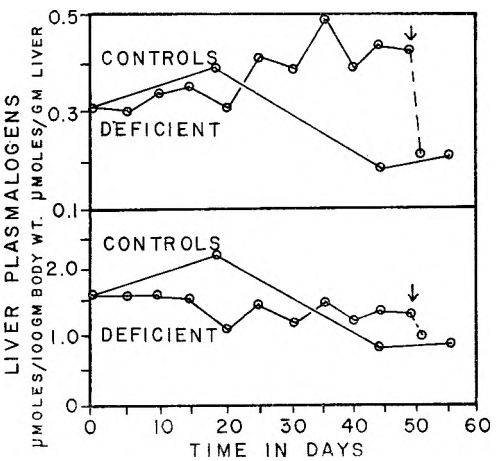

Fig. 4 Change in liver plasmalogens *versus* time, in rats fed the control and thiamine-deficient rations. One milligram of thiamine was injected into each deficient rat at the arrow.

thiamine was injected on day 49, a significant drop in phospholipids per gram of liver occurred, while with respect to total body weight, a significant increase occurred.

As shown in figure 4, the plasmalogens followed much the same pattern as the phospholipids, with the exception that they were held most tenaciously, remaining remarkably constant with respect to body weight. Per gram of liver a significant increase occurred as thiamine deficiency developed. Again, as with total phospho-

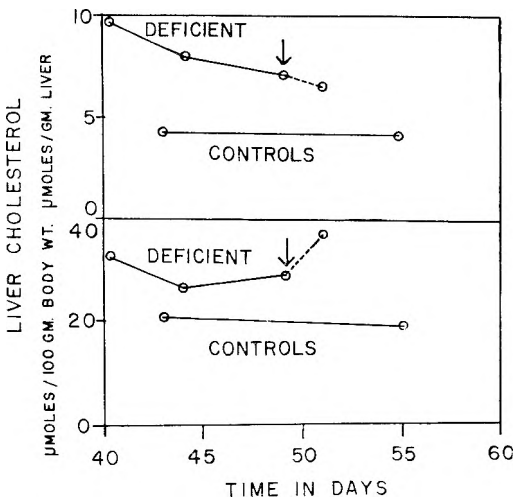

Fig. 5 Change in liver cholesterol after 40 days of feeding the control and thiamine-deficient rations. One milligram of thiamine was injected into each deficient rat at the arrow.

lipids, a significant change occurred when thiamine was injected with a rapid return to the normal control level.

Originally, total cholesterol was determined to see if the slight rise in total lipids per 100 gm of body weight beginning on day 39 could be accounted for by a change in cholesterol, since the change was not accounted for by phospholipid. The cholesterol values, however, (fig. 5) followed more nearly the pattern of the phospholipids than of the total lipids. With respect to body weight after thiamine injection, cholesterol rose sharply to almost double the normal control level.

DISCUSSION

The dramatic response of total liver lipids to thiamine injection indicates that the actual deposition of lipid in the liver is controlled very strongly by thiamine. This increase in liver lipid is due almost solely to the deposition of neutral lipid as opposed to phospholopid. As the thiamine deficiency developed, it appears that neutral lipids were exhausted from the liver to serve as an energy supply. Once this supply was depleted, the animals began to die from the vitamin deficiency. The first animals died from the deficiency on day 46. It was just at this time that total lipids were the most depleted. From the results after thiamine was injected, it appears that one of the important functions of thiamine is to allow repletion of liver lipid at a very rapid rate. The source of this lipid cannot be ascertained quantitatively from these experiments. However, from the iodine number of the new lipid, it is considerably more saturated than the lipid of the depleted animals. It is also considerably less than the iodine number of the corn oil that was fed, indicating that the new lipid arises from synthesis of saturated fatty acids. The food intake over the 48-hour period after thiamine injection was not substantially higher than during the deficiency period. This indicates also that increased food intake does not explain the rapid redeposition of lipid in the liver.

The phospholipids were lost at the same rate as general liver substance but at a slightly faster rate than body weight. The drop in phospholipids per gram of liver when thiamine was injected can probably be attributed to the lack of stimulation of phospholipid synthesis coupled with increased deposition of neutral lipid and other liver components.

The original idea that the long-chained aldehydogenic component of the plasmalogens may arise from decarboxylation of long-chain α-keto acids is not substantiated by the present experiments. There was no tendency for a decreased synthesis of the plasmalogens. These results in general substantiate those obtained by Anchel and Waelsch ('43) for nervous tissue and muscle. In fact, they are held very tenaciously by the liver, even more so than the phospholipids in general. The source of the aldehyde-yielding component is still open to question, and apparently from the present experiments thiamine does not appear to be involved in its production. The sudden decrease in plasmalogen concentration when thiamine was injected can probably be explained on the same basis that phospholipid concentration decreased after thiamine injection, namely, the lack of stimulation of plasmalogen synthesis by thiamine coupled with increased deposition of neutral lipid and other liver components.

In the thiamine-deficient rats, liver cholesterol was higher both when expressed as concentration per gram of liver and as

concentration per 100 gm of rat. The significance of this is not evident unless it can be assumed that the cholesterol content of the liver at the later stages of thiamine deficiency consists of firmly bound and metabolically active cholesterol. Thus as labile liver components are lost as the deficiency progresses, the cholesterol necessary for maintaining integrity of the cell remains and shows up as a higher cholesterol concentration than the normal controls. When thiamine was injected, the cholesterol concentration per gram of liver decreased slightly probably for the same reason that phospholipids and plasmalogens decreased. When calculated in terms of body weight, cholesterol increased sharply when thiamine was injected. The significance of this change is not apparent at present. This was the only change observed of all the components studied that did not tend to return to the normal control value when thiamine was injected. In view of the importance of cholesterol in various human lipid disorders, this interrelationship of thiamine and cholesterol should be studied further. In fact, the sudden response of all liver lipids to the introduction of thiamine may have importance in the treatment of many diseases in which thiamine therapy is employed.

SUMMARY

The response of total liver lipids, phospholipid, plasmalogens, and cholesterol to thiamine deficiency has been studied in the rat. As expected, neutral lipid, except for cholesterol, fell rapidly to well below the normal control levels. Phospholipids were mainly unaffected during the deficiency, and plasmalogens showed a tendency to be maintained at a high level regardless of the deficiency. Cholesterol was higher in thiamine-deficient rat liver than in the normal controls. The sudden reintroduction of thiamine by injection caused total lipids to rebound to a high normal level and cholesterol, when expressed in terms of body weight, to reach a level almost twice that of normal. Phospholipids and plasmalogens followed patterns after thiamine injection that can be explained in terms of the maintenance of important cellular components that resist dietary changes.

LITERATURE CITED

Anchel, M., and H. Waelsch 1943 Concentration of higher fatty aldehydes in tissues of vitamin-B deficient rats. Federation Proc., *2:* 57.

Arnold, A., and C. A. Elvehjem 1939 Influence of the composition of the diet on the thiamine requirement of the dog. Am. J. Physiol., *126:* 289.

Basford, R. E. 1958 A lipoprotein obtained from succinic dehydrogenase complex. Federation Proc., *17:* 186.

Boxer, G. E., and DeW. Stettin, Jr. 1944 The role of thiamine in the synthesis of fatty acids from carbohydrate precursors. J. Biol. Chem., *153:* 607.

Engel, R. W., and P. H. Phillips 1939 Fatty livers as a result of thiamine administration in vitamin B_1 deficiency in the rat and the chick. J. Nutrition, *18:* 329.

Feulgen, R., and K. Voit 1924 Über einen weitverbreiteten festen Aldehyd. Pflüger's Arch. ges. Physiol., *206:* 389.

Feulgen, R., W. Boguth and G. Andresen 1951 Quantitative bestimmung der acetalphosphatide (plasmalogens). Z. physiol. Chem., *287:* 90.

Fiske, C. H., and Y. SubbaRow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

Gavin, G., and E. W. McHenry 1940 The B vitamins and fat metabolism. III. The effects of vitamin B_6 upon liver and body fat. Ibid., *132:* 41.

Green, D. E., W. W. Westerfeld, B. Vennesland and W. E. Knox 1942 Carboxylases of animal tissues. Ibid., *145:* 69.

Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. Ibid., *138:* 459.

Hornykiewytsch, T., and G. Geydl 1951 Histochemische und serologische untersuchungen über die wirkung der röntgenstralhens. I. Einleitung problemstellung, chemie der untersuchten stoffe, methodie. Strahlentherapie, *84:* 481.

Joel, C. D., M. L. Karnovsky, E. G. Ball and O. Cooper 1958 Lipide components of the succinate and reduced diphosphopyridine nucleotide oxidase system. J. Biol. Chem., *233:* 1565.

Marinetti, G. V., J. Erbland and E. Stotz 1958 The structure of pig heart plasmalogens. J. Am. Chem. Soc., *80:* 1624.

——— 1959 The structure of beef heart plasmalogens. Ibid., *81:* 861.

McHenry, E. W. 1935 An effect of choline upon the weights of young rats. J. Physiol., *85:* 343.

——— 1937 Vitamin B_1 and fatty livers. Ibid., *89:* 287.

McHenry, E. W., and G. Gavin 1938 The B vitamins and fat metabolism. I. Effect of thiamine, riboflavin and rice polish concentrate upon body fat. J. Biol. Chem., *125:* 653.

Pearson, S., S. Stern and T. H. McGavack 1953 A rapid, accurate method for the determination of total cholesterol in serum. Anal. Chem., *25:* 813.

Rapport, M. M., B. Lerner, N. Alonzo and R. E. Franzl 1957 The structure of plasmalogens II. Crystalline lysophosphatidyl ethanolamine (acetal phospholipide). J. Biol. Chem., 225: 859.

Rubino, F., M. C. Ciancimino and O. Giacalone 1956 Influenza della vitamina B_1 sul contenuto corporeo in acqua, in azote totale e in lipidi di ratti realimentati dopo digiuno. Bull. Soc. ital. Biol. sper., 32: 36.

Williams, J. N., Jr., and C. A. Elvehjem 1949 The relation of amino acid availability in dietary protein to liver enzyme activity. J. Biol. Chem., *181:* 559.

Yarbro, C. L., and C. E. Anderson 1956 Acetal phosphatides in the adipose tissue of newborn rats. Proc. Soc. Exp. Biol. Med., *91:* 408.

——— 1957 Acetal phosphatides in adipose tissue and liver of starved rats. Ibid., 95: 556.

Production of Low-Fat Milk

I. EFFECT OF QUALITY AND QUANTITY OF CONCENTRATE ON THE VOLATILE FATTY ACIDS OF THE RUMEN AND ON THE COMPOSITION OF THE MILK[1]

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It was first reported by Powell ('38, '39, '41) that the fat content of the milk of cows could be depressed as much as 60% by feeding low-roughage rations or by feeding the roughage in a finely ground state. He also reported that the fat content of the milk of these cows increased when the concentrates fed were first fermented with rumen material and he proposed a relationship between the activities in the rumen and the composition of milk. No data were presented. Others who have observed a relation between the roughage content of the ration and the percentage of fat in milk are Loosli et al. ('45), McClymont ('50), Stoddard et al. ('49), and Balch et al. ('52). The latter two groups of workers noted that the depression of the fat content of milk by low-roughage rations was associated with a lowered proportion of acetate and an increased proportion of propionate in the rumen. Balch et al. ('55) observed that flaked maize, fed with low levels of hay, depressed the fat content of milk more than a ration containing crushed oats and barley. It has been the experience of the authors in repeated trials that it is difficult to reproduce consistently the results reported by Powell and others when the more commonly used concentrate mixtures are employed.

The principal objective of this study was to develop rations which would effect a decrease in the fat content of milk more consistently than had been effected by the usual low-roughage—high-concentrate rations and which could be used in further studies of the factors which control milk fat secretion. Concurrently information was obtained on rumen volatile fatty acids (VFA), solids-not-fat of milk and the nature of the milk fat. A brief summary of some of these data has been reported by Shaw et al. ('57).

EXPERIMENTAL

Three feeding trials were conducted during the years 1951, 1955 and 1956 with a total of 25 lactating cows. These cows were in their second to 5th months of lactation and consisted of two Jerseys, 7 Guernseys, 12 Holsteins and 4 Ayrshires. In trials 2 and 3 in which group comparisons were made, the different groups were matched as evenly as possible on the basis of milk production, stage of lactation, body weight and previous average milk fat. The rations fed are shown in table 1. The digestible protein and total digestible nutrient (TDN) content of the various rations were calculated from Morrison's ('48) tables. U. S. no. 2 alfalfa hay was used in all of the feeding trials.

In the first trial normal feeds were used. Four lactating cows which had been on regular herd rations were maintained for two weeks on a regulated diet consisting of 15 pounds of alfalfa hay daily plus concentrate mixture no. 1. They were then changed to three or 4 pounds of this hay per day plus increased amounts of the same concentrate. Two of the cows were changed from these low levels of hay to equal amounts of pelleted, dehydrated alfalfa meal during the last 20 days of the feeding trial. TDN intake was maintained at approximately 100% of Morrison's ('48)

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TABLE 1
Concentrate mixtures

Ingredients	Mixture number						
	1	2	3	4	5	6	7
	lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*
Bread, partially dried	—	—	100	—	—	45	45
Potato meal	—	—	—	—	—	20	20
Cooked polished rice	—	—	—	—	—	20	20
Dried skimmilk (low fat)	—	—	—	—	—	—	25
Corn meal	380	550	—	2500	200	—	—
Hominy feed	480	—	—	—	—	—	—
Crimped barley	—	—	—	—	1160	—	—
Distillers' dried grains	100	—	—	—	—	—	—
Brewers' dried grains	100	—	—	—	—	—	—
Crimped oats	400	—	—	—	—	—	—
Citrus pulp	—	330	—	1600	300	—	—
Wheat bran	300	—	—	—	—	—	—
Molasses	—	200	[1]	500	200	15	15
Linseed meal	200	—	—	—	—	—	—
Soybean oil meal	—	1100	—	—	100	—	—
Dicalcium phosphate	20	20	—	50	20	—	—
Salt	20	20	—	50	20	—	—
Digestible protein, %[2]	15.1	20.2	9.1	4.4	11.5	6.0	11.0

[1] One pound of molasses per cow per day.
[2] Calculated from Morrison's ('48) tables.

recommended allowances during the two-week preliminary period, a 4-day change-over period and a 52-day experimental period.

For the second trial, 4 groups of three cows each were used. During a preliminary period of two weeks the cows were maintained on 12 pounds of alfalfa hay plus a sufficient amount of concentrate mixture no. 2 to equal approximately 110% of Morrison's recommended TDN allowances; this level of TDN was fed throughout the entire experiment. During the succeeding 6 days, cows were changed gradually to the experimental rations and continued on these rations for 91 days. The experimental rations received by the 4 groups were as follows: group I, a high-protein concentrate (mixture no. 2) plus 4 pounds of alfalfa hay daily; group II, white bread plus 6 or 7 pounds of alfalfa hay and one pound of blackstrap molasses daily (bread was fed because of field observations implicating bread in connection with low milk fat); group III, a low-protein concentrate (mixture no. 4) plus 4 pounds of alfalfa hay daily; group IV, a low-protein concentrate (mixture no. 4) plus 12 pounds of alfalfa hay daily.

For the third trial, three groups of cows each received 15 pounds of alfalfa hay daily plus sufficient concentrate mixture no. 5 to maintain the TDN intake at approximately 110% of Morrison's recommended allowances. After being fed this control ration for 7 weeks the cows were changed gradually during a two-week period to rations consisting of concentrate mixtures made up of cooked or heated high-starch, low-fat feeds. The cows were continued on these rations for 4 weeks beyond the two-week change-over period. These rations consisted of 6 pounds of alfalfa hay daily per cow plus concentrate mixture no. 7 for group I and concentrate mixture no. 6 for groups II and III. The TDN intakes during this period were maintained at approximately 115, 75 and 115% of calculated allowances for groups I, II and III respectively. The principal variations, therefore, were in energy intake and protein levels.

In each of the feeding trials discussed in this paper the cows were weighed at the beginning of the studies and at two-week intervals thereafter. Daily milk production was recorded. The fat content of the milk was determined (Babcock procedure) during the last 6 days of the preliminary periods and thereafter on alternate days in the first trial, daily in the second trial and for three consecutive days

TABLE 2

Effect of feeding low-roughage (pelleted and non-pelleted) plus a high concentrate ration[1] upon production of milk and milk fat (trial 1)

Cow	Normal,[2] 14 days	Change,[3] 4 days	1st week	2nd[4] week	3rd week	4th week	5th[5] week	6th week	7th week
				Milk fat, %					
4–G	4.95	4.50	4.25	4.28	4.15	4.40	4.90	5.10	5.50
5–G	4.40	4.10	4.20	4.47	4.65	4.65	4.40	4.50	4.50
6–G	4.80	4.86	4.18	3.98	4.35	4.70	4.90	5.10	5.00
7–J	5.70	5.55	5.30	5.63	5.55	5.60	6.00	6.00	6.20
Av.	4.97	4.74	4.47	4.56	4.64	4.80	5.06	5.20	5.18
				Av. daily milk production in pounds					
4–G	22.4	20.5	22.2	21.7	21.7	20.2	17.5	17.1	16.4
5–G	25.2	24.2	19.6	20.5	19.8	18.2	18.4	16.4	16.2
6–G	23.0	23.0	22.1	22.6	23.0	21.0	20.9	20.5	20.6
7–J	22.3	20.8	20.5	19.4	19.1	18.4	18.4	14.3	14.1
Av.	23.2	22.1	21.1	21.1	21.0	19.4	18.8	17.1	17.1

[1] Concentrate mixture no. 1.

[2] Data represent average of last three days of periods.

[3] Cows 4–G, 5–G and 7–J changed from 10 pounds to 4 pounds and cow 6–G to 3 pounds alfalfa hay per day plus increased concentrate.

[4] Cows 4–G and 5–G changed to 3.5 pounds hay and cow 7–J to 3 pounds alfalfa hay per day. Cow 6–G continued at 3 pounds.

[5] Cows 5–G and 7–J fed 3.5 pounds and 3 pounds, respectively, of pelleted alfalfa meal as sole roughage for remainder of experiment.

every two weeks in the third trial. The latter interval was adopted in the third trial because the data from trials 1 and 2 showed that such frequent testing was unnecessary.

Adjustments in TDN intake were made every week in trials 1 and 2 and every two weeks in trial 3. The changes to be made for each animal were calculated on the basis of the average daily milk production and milk fat content of the previous period (one or two weeks) and the last body weight obtained.

During trial 2, rumen fluid was obtained from each cow on the 5th day of each week from the 6th through the 10th week after the change-over periods. From 750 to 1000 ml of rumen fluid was obtained 4½ to 5½ hours after feeding by means of a large rumen tube (Colorado tube) and aspirator. Immediately after removal, a sample of the fluid was strained through cheese cloth and 1 ml of saturated mercuric chloride was added per 39 ml of fluid as a preservative. The rumen volatile fatty acids (VFA) were determined in duplicate by the Keeney chromatographic procedure ('56b).

For simplicity in presenting the results the VFA will be referred to as acetic, butyric, propionic, valeric, and caproic acids although the Keeney technique does not separate the various isomers which are known to exist (El-Shazley, '52; Annison, '54).

All of the milk produced was collected from each animal during the first three days of periods 8, 9 and 10 of trial 2. The milk fat obtained by churning the cream was purified and the iodine number determined by the Rosenmund and Kuhnhenn method ('23) and butyric acid by the Keeney method ('56a). Milk solids-not-fat were determined by the Watson procedure ('56).

RESULTS

I. Total milk production and fat and solids-not-fat content of milk

Trial 1. From the results obtained during the first trial (table 2) it will be noted that a truly marked decrease in the fat content of the milk was not achieved by the feeding of a high level of a commonly used concentrate mixture plus 3.0 to 3.5 pounds per day of either alfalfa hay or pelleted alfalfa meal. The milk production data and the body weights reflected the adequate level of TDN intake which was maintained. The 4 cows gained an average of

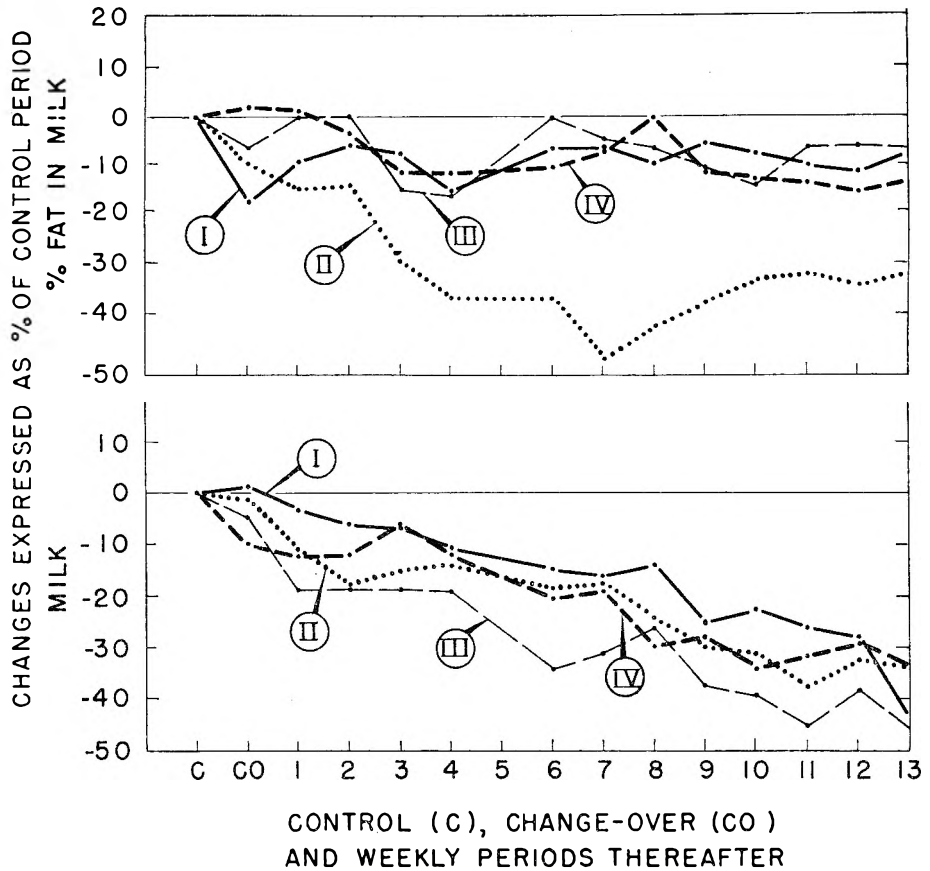

Fig. 1 Effect of kind of concentrate and amount of hay on milk production and fat content of milk. The daily feeding regimen was as follows: group I, high-protein concentrate + 4 pounds hay; group II, bread + molasses + 6–7 pounds hay; group III, low-protein concentrate + 4 pounds hay; group IV, low-protein concentrate + 12 pounds hay.

13.5 pounds during the experimental period.

Trial 2. In the second trial, as will be noted in figure 1, three cows in group II which received large amounts of bread (14 to 22 pounds daily) exhibited a decrease of over 30% in the fat content of the milk for a period of two months. Only slight decreases occurred in the fat content of the milk of the cows in the other three groups. It is significant that two cows in group II ("bread group") received 6 pounds of hay daily and the other, a larger animal, received 7 pounds, whereas the cows in the other three groups received only 4 pounds per day. The differences in the decrease in the milk fat content between group II and the other groups were found by analysis of variance to be highly significant. No statistically significant differences in milk fat were noted between group I (high-protein concentrate, low-roughage) and group III (low-protein concentrate, low-roughage) or between group III (low-protein concentrate, low-roughage) and group IV (low-protein concentrate plus 12 pounds of hay).

The actual TDN intakes of the cows in groups I to IV during the 91-day experimental period were calculated to be 115, 101, 107 and 111%, respectively, of Morrison's suggested TDN allowances. The average body weight changes in pounds during this period for groups I to IV were +67, +51, −37 and 0. The protein requirements were amply provided for in groups I and II whereas groups III and IV consumed approximately 70 and 90% of Morrison's recommended allowances. The

changes in milk production shown in figure 1 are expressed as a percentage of the production during the preliminary period. Group I maintained production at a somewhat higher level than the other groups, probably due to the maintenance of a more uniform and slightly higher energy intake. The cows in group III decreased in milk production more than the other groups probably due to their low-protein intake.

Trial 3. The data on the milk production of the cows fed the low-roughage rations made up primarily of heated concentrates are presented in graphic form in figure 2. Groups I and III maintained milk production equally well, whereas, that of group II fell more rapidly, probably due to the low protein intake. Group II on the low-protein, high-energy regimen exhibited the greatest decrease in the fat content of the milk (33 to 40%), whereas group II, on the same ration, but on a low-energy intake, exhibited no decrease in the fat content of the milk. Group I, on the ration with the added skim milk as a source of protein, also exhibited a decrease in the fat content of the milk but much less than group III.

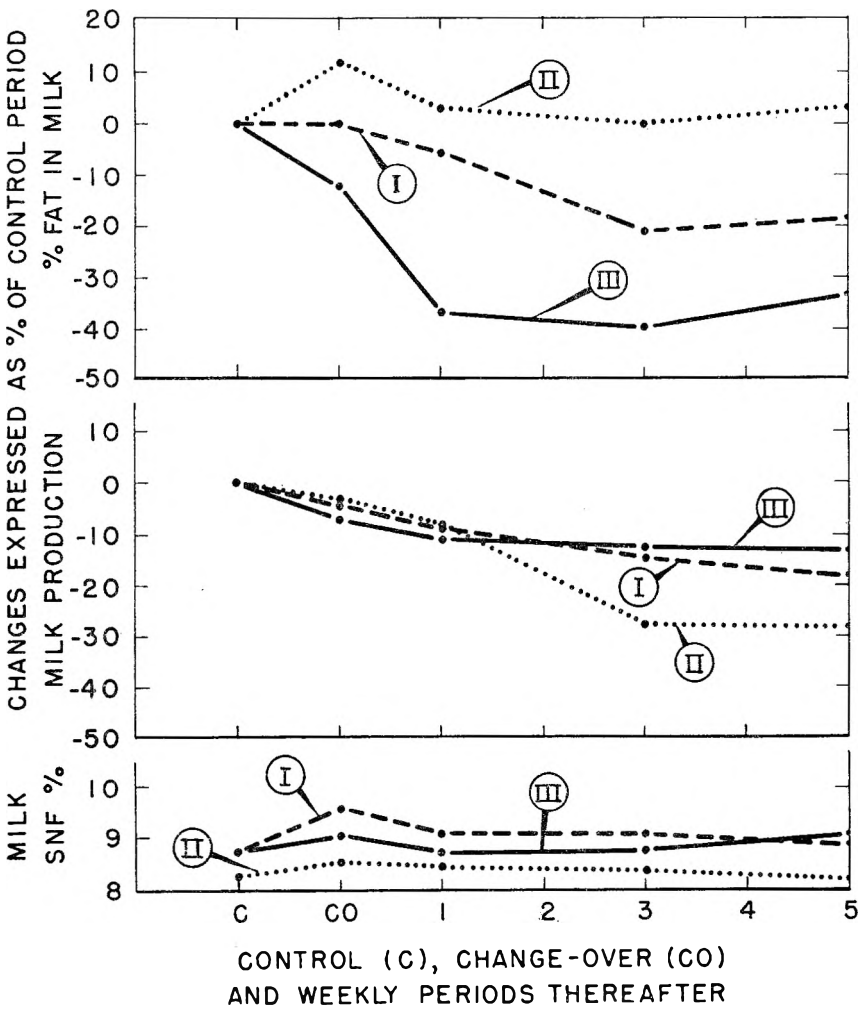

Fig. 2 Effect of feeding heated concentrates plus low roughage on the fat content of milk. The feeding regimen was as follows: group I, high-protein, high-energy intake; group II, low-protein, low-energy intake; group III, low-protein, high-energy intake.

TABLE 3

Rumen voatile fatty acids[1] (VFA) and butyric acid and iodine number of milk fat[2] of cows on "high-bread" and other high-concentrate, low-roughage rations (trial 2)

	Group[3] (3 cows each)	Total Rumen VFA	Rumen VFA as molar % of total					Butyric acid in milk fat	Iodine no. of milk fat
			Acetic	Propionic	Butyric	Valeric	Higher		
		μM/ml						*molar %*	
I	HPC + 4 pounds hay	147.9 ± 26.6	58.3	20.8	15.9	3.9	1.1	8.0 ± 0.6	33.3 ± 3.5
II	Bread + 6–7 pounds hay	116.8 ± 28.7	52.0	26.0	14.3	5.4	2.6	6.8 ± 1.5	35.2 ± 5.9
III	LPC + 4 pounds hay	90.7 ± 4.0	61.7	16.9	16.7	3.1	1.5	9.8 ± 0.8	33.7 ± 3.9
IV	LPC + 12 pounds hay	101.3 ± 14.3	65.3	17.8	13.5	2.2	1.1	9.1 ± 0.6	34.7 ± 2.4

[1] Means of 15 observations per group.
[2] Means of 9 observations per group.
[3] HPC—high-protein concentrate. LPC—low-protein concentrate.

TABLE 4

Analysis of variance of the data on VFA (as molar % of total) in rumen fluid (trial 2)

Source of variance	Degrees of freedom	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Higher acids
		m.s.[1]	m.s.	m.s.	m.s.	m.s.
Between groups	3	477.8[2]	249.9[2]	32.3[2]	26.6[2]	7.2[2]
Between periods	4	22.7	6.7	9.6	1.2	1.7
Group × periods	12	21.0	6.8	3.2	2.3	1.2
Cows in groups	8	37.8	46.2	6.2	2.9	1.4
Cows × periods	12	31.5	17.1	2.3	2.2	0.9

[1] m.s.—mean square.
[2] $P = 0.01$.

The graphic data in figure 2 fail to disclose any significant variations among the three groups in the milk solids-not-fat. Thus the solids-not-fat content of the milk was maintained as well by the diets made up predominately of heated concentrates as by the more normal diets.

II. Rumen volatile fatty acids

Trial 2. In the following discussion the values given for the individual VFA represent molar per cent of total VFA in the rumen fluid. The average rumen VFA for each group for the weekly periods 6 through 10 are shown in table 3. Each value represents a total of 15 rumen samples (5 from each animal). The analyses of variance of these data are shown in table 4. Using the variance ratio it was established that the sole source of significant variance was due to differences between groups. Duncan's test ('55), which provides very stringent criteria for estimating the significance in the differences between group means, was used to estimate the significance of these data.

For acetic acid, the 4 groups were in the decreasing order of IV, III, I and II and all differences between groups were significant ($P = 0.01$).

The propionic acid of group II was significantly higher than all others ($P = 0.01$) and that of group I was significantly higher than that of groups III and IV ($P = 0.01$). Groups III and IV were not significantly different from each other in this respect.

The butyric acid values for groups II and IV were significantly lower ($P = 0.01$) than in the other two groups but they did not differ significantly from each other. The butyric acid of group III was the highest and was significantly higher than that of groups II and IV ($P = 0.01$) and of group I ($P = 0.05$).

The valeric acid of group II was significantly higher than in all others ($P = 0.01$); group I was significantly higher than group IV ($P = 0.01$) but not higher than group III, and group III was significantly higher than group IV ($P = 0.05$).

For hexaenoic and higher acids, group II was higher than all others ($P = 0.01$) but the others were not significantly different from each other.

Thus, group II which exhibited such a marked decrease in fat content of milk and the lowest level of butyric acid in the milk fat, showed the greatest changes in the molar proportions of rumen acetic, propionic, valeric and caproic acids. The actual decreases in the fat content of milk (expressed as a percentage of the fat content during the normal periods) between the control period and the average of periods 6 through 10 (rumen sample periods) were 7.1, 38.3, 7.4 and 8.4% respectively for groups I, II, III and IV. The reduction of the fat content was significantly related to the reduction in acetate ($r = +0.64$, $P < 0.01$) and inversely related to the increase in propionate ($r = -0.63$, $P < 0.01$). The absolute concentrations of the various rumen VFA were so obviously not related to the changes in the fat content of milk that the data are not included in these tables. Group III, which received the least protein, had the highest molar proportion of butyric acid in the rumen.

III. Butyric acid content and iodine number of milk fat

The butyric acid content (table 3) of the milk fat of the cows in group II (periods 8, 9 and 10) was below normal and appreciably lower than that of the other groups. That of group I was lower than that of groups III and IV. It will also be noted that the iodine numbers of the different groups for periods 8, 9 and 10 did not differ appreciably. Indeed, in view of the differences in the butyric acid content of the milk fat the small differences in iodine number were unexpected.

DISCUSSION

It is evident that the nature of the concentrate is of great importance in inducing alterations in the fat content of milk. For example, cows receiving a ration consisting predominantly of bread and 6 or 7 pounds of hay exhibited much greater decreases in the fat content of milk than those receiving only 4 pounds of hay and a concentrate mixture of cereals and cereal by-products more commonly used in mixed concentrates for dairy cattle. The marked decreases in the fat content of milk produced by the cooked high-starch concentrate mixtures of polished rice, potato meal, bread and molasses, and with the high-bread ration, are believed to be due to alterations in the starch induced by heating and is reminiscent of the study by Balch et al. ('55) with flaked maize.

In view of the correlation coefficients between the molar percentages of the rumen VFA (as percentage of total VFA) and changes in the fat content of milk, it is unlikely that alterations in the molar proportions of rumen fluid butyrate were responsible for the changes in milk fat content; a reduction in the molar proportion of acetate or an increase in the molar proportion of propionate can therefore be considered to be implicated in this phenomenon. The high positive correlations between rumen acetate and the percentage of fat in milk and the equally high negative correlations between rumen propionate and the percentage of fat in milk (expressing these VFA as molar per cent of total VFA) offer some interesting possibilities. For example it appears that the analysis of rumen fluid for VFA may be a valuable guide for the evaluation of dietary regimens for their probable effect on the fat content of milk.

It has been demonstrated by means of C^{14}-labeled acetate (Popjak et al., '51) and β-hydroxybutyrate (Shaw and Lakshmanan, '57) that these two substances are important blood precursors of the lower fatty acids of milk fat of ruminants. β-Hydroxybutyric acid is the principal component of the acetone bodies in the blood of the cow (Knodt et al., '42).

It is becoming evident that the synthesis of the lower fatty acids by the udder may exert a controlling influence on the fat content of milk (Shaw and Lakshmanan, '57). The possible significance, in relation to the decrease in milk fat production, of the large increase in the proportion of propionic acid and of valeric and higher acids is not known. It is believed that the increased production of propionate may play an important role, due to its antiketogenicity, by decreasing the formation of β-hydroxybutyrate by the liver. Earlier, Shaw and Lakshmanan ('57) proposed that the synthesis of the VFA of milk fat from acetate and β-hydroxybutyrate represents the bulk of the lipogenic efforts of the lactating udder. It is suggested that the decreased fat content of milk observed during the feeding of diets consisting primarily of cooked concentrates is due to a deficiency of both major blood precursors (acetate and β-hydroxybutyrate) of the VFA of milk fat, acetate due to an actual decrease in relative production in the rumen and β-hydroxybutyrate due to the antiketogenicity of propionate.

It is perhaps significant that the largest decrease in the fat content of milk which was observed in trial 2 was that of group II in which the most marked changes in the proportions of rumen VFA were noted. It is possible that the changes in the rumen VFA were of sufficient magnitude to depress the VFA of milk fat and thus the fat content of the milk; this is in contrast to the results in group I in which there were significant but smaller changes in the rumen VFA as compared to those in groups III and IV and a much smaller decrease in the butyric acid of milk fat and in the percentage of milk fat.

In several instances, in contrast to reports by El-Shazly ('52), Davis et al. ('57) and Balch and Rowland ('57), the molar proportion of rumen butyric acid was not related to the protein content of the ration in comparisons between very high and very low levels of protein intake.

A decrease in the Reichert-Meissl value and an increase in the iodine number of the milk fat of cows on low-roughage rations has been reported by Stoddard et al. ('49), McClymont ('52) and Balch et al. ('52, '55). However, from the data given on the energy intake in these studies it is difficult to determine whether the changes in the fat constants were due specifically to the low-roughage ration or to underfeeding; the latter is known to produce such changes in the milk fat constants (Eckles and Palmer, '16). In the present studies, where energy intake was maintained at or above calculated requirements, the decrease in the butyric acid content of the milk fat was not accompanied by an increase in the degree of unsaturation, such as occurs with fasting, and therefore the decreases noted in the butyric acid content of the milk fat do not appear to have been due to fasting. It is significant that the iodine numbers of the milk fat did not differ appreciably among the different groups. This suggests that an increase in the degree of unsaturation is not necessarily associated with the changes in the fat content of the milk induced by certain alterations in the ration which decrease the VFA content of milk fat. This is in accord with the concepts of milk fat synthesis proposed by Shaw and Lakshmanan ('57).

CONCLUSIONS

1. Cows receiving rations made up primarily of cooked high-starch feeds produced milk with a low-fat content. Cows receiving the more commonly-fed concentrates with even lower levels of roughage produced milk with only slight decreases in the fat content.

2. A ration composed primarily of bread effected a decrease of more than 30% in the fat content of milk and a low level of butyric acid in the milk fat but had little effect on the iodine number of the milk fat. In terms of molar per cent of total rumen volatile fatty acids there was a positive correlation (+0.64) between the fat content of milk and rumen acetic acid and a negative correlation (−0.63) between the fat content of milk and rumen propionic acid.

3. A ration composed primarily of cooked rice, cooked potato meal, bread and molasses effected a marked decrease in the fat content of milk. This effect was prevented by maintaining a low-energy intake and ameliorated to a considerable extent by adding dried skim milk to the ration.

4. Milk solids-not-fat remained relatively uniform regardless of diet.

LITERATURE CITED

Annison, E. F. 1954 Some observations on volatile fatty acids in the sheep's rumen. Biochem. J., *57:* 400.

Balch, C. C., D. A. Balch, S. Bartlett, C. P. Cox and S. J. Rowland 1952 Studies of the secretion of milk of low fat content by cows on diets low in hay and high in concentrates. I. The effect of variations in the amount of hay. J. Dairy Res., *19:* 39.

Balch, C. C., D. A. Balch, S. Bartlett, Z. D. Hasking, V. W. Johnson, S. J. Rowland and J. Turner 1955 Studies of the secretion of milk of low fat content by cows on diets low in hay and high in concentrate. V. The importance of the type of starch in the concentrates. Ibid., *22:* 10.

Balch, D. A., and S. S. Rowland 1957 Volatile fatty acids and lactic acid in the rumen of dairy cows receiving a variety of diets. Brit. J. Nutrition, *11:* 288.

Davis, E. F., N. S. Woodhouse, M. Keeney and S. H. Beck 1957 The effect of various levels of dietary protein upon the volatile fatty acids in the rumen of the dairy cow. J. Dairy Sci., *40:* 75.

Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.

Eckles, C. H., and L. S. Palmer 1916 The influence of the plane of nutrition of the cow upon the composition and properties of milk and butter fat. Influence of underfeeding. Mo. Agr. Exp. Sta. Bull., *25:* 1.

El-Shazly, K. 1952 Degradation of protein in the rumen of the sheep: 1. Some volatile fatty acids, including branched chain isomers formed *in vivo*. Biochem. J., *51:* 640.

Keeney, M. 1956a A survey of United States butterfat constants 11. Butyric acid. J. Assoc. Off. Agric. Chemists, 39: 212.

——— 1956b Direct chromatographic determination of C_1 to C_6 fatty acids in rumen fluid. Md. Agr. Exp. Sta. Misc. Pub. no. 238, p. 23.

Knodt, C. B., J. C. Shaw and G. C. White 1942 Studies on ketosis in dairy cattle. I. Effect of stall and pasture feeding upon the concentration of blood and urinary acetone bodies of dairy cattle. J. Dairy Sci., 25: 837.

Loosli, J. K., H. L. Lucas and L. A. Maynard 1945 The effect of roughage intake upon the fat content of milk. Ibid., 28: 147.

McClymont, G. L. 1950 The relation of the type and quantity of roughage and grazing to the fat content of milk. Australian Vet. J., 26: 111.

Morrison, F. B. 1948 Feeds and Feeding, 21st ed. The Morrison Publ. Co., Ithaca, N. Y.

Popjak, G., T. H. French and S. J. Folley 1951 Utilization of acetate for milk fat synthesis in the lactating goat. Biochem. J., 48: 411.

Powell, E. B. 1938 One cause of fat variation in milk (Abs.). Am. Soc. Animal Prod. Proc., p. 40.

——— 1939 Some relations of roughage intake to the composition of milk (Abs.). J. Dairy Sci., 29: 453.

——— 1941 Progress report on the relation of the ration to the composition of Milk (Abs.). Ibid., 24: 504.

Rosenmund, K. W., and W. Kuhnhenn 1923 Eine neue methode zue jodzahl bestimmung in fetten und olen unter verevendung von pyridinsulfatdibromid. Ztschr. Untersuch. Nahr. u. Genuss., 46: 154.

Shaw, J. C., and S. Lakshmanan 1957 Lactation and hormones. Atomic Energy and Agriculture, AAAS, Symposium Volume no. 49.

Shaw, J. C., R. R. Robinson, M. E. Senger, E. C. Leffel, R. N. Doetsch, T. R. Lewis and W. H. Brown 1957 Ruminant metabolism on diets producing a low fat content milk. Md. Agr. Exp. Sta. Misc. Pub. no. 291: 16.

Stoddard, G. E., N. N. Allen and W. H. Patterson 1949 Some effects of a low roughage, high concentrate ration on the fat of cow's milk (Abs.). J. Animal Sci., 8: 630.

Watson, P. D. 1956 A Lactometer Method for Determining the Solids in Milk. Eastern Utilization Research Branch, Agricultural Research Service, USDA, Washington, D. C. ARS-73-10.

Repletion and Depletion of Polyunsaturated Fatty Acids in Cebus Monkeys[1]

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The composition of the dietary fat influences the fatty acid composition of tissue lipids in many species including man (Deuel, '57). The nature of the dietary fat also influences the concentrations of cholesterol in the blood (Ahrens, '57). There is, however, no good evidence that these two observations are causally related. It has been consistently observed in studies on animals and humans that when the type of dietary fat is changed the observed increases or depressions in the serum cholesterol level occur in a very brief period, usually within two weeks or less. Relatively much less information is available about the sequence of changes of concentrations of polyunsaturated fatty acids (PFA) in various tissues and in different classes of lipids in those tissues. Such information would seem to be important to an understanding of the mechanisms by which PFA influence the concentrations of cholesterol in the blood.

The Cebus monkey, which has been used extensively in this laboratory for studies of experimental atherosclerosis (Mann et al., '53), has very different serum cholesterol concentrations when different fats are fed (Portman et al., '56). Similarly, the feeding of different types of fats results in different levels of PFA in the total serum lipid fraction and in the cholesterol ester fraction (Portman et al., '59).

The present study was undertaken to evaluate the effect of feeding diets very low and very high in PFA on the PFA concentrations in lipids of the sera and other tissues of Cebus monkeys and on the rates at which these concentrations change when the monkeys are transferred from one extreme diet to the other.

METHODS

Twenty-two cinnamon Cebus monkeys were used during the various phases of this experiment. They weighed between 1300 and 1900 gm and were housed and fed as previously described (Mann et al., '53; Portman et al., '56). Each monkey was offered a daily ration containing 400 Cal. and 17 gm of vitamin-free casein.[2] The ration was offered in the morning and was invariably consumed (with a certain undefined amount of wastage) within two hours.

Samples of blood and tissue were obtained in the morning just prior to offering the daily ration except in certain cases where samples were taken at fixed periods after the initiation of feeding. Serum was obtained from the clotted blood within 45 minutes after venipuncture. One milliliter of sera was added to a 25-ml volumetric flask and 20 ml of chloroform-methanol (2: 1 v/v) were added with swirling. After heating the flasks in a 40° water bath for 20 minutes and cooling to room temperature, the flasks were brought to the mark with chloroform-methanol. The material was then filtered into large cul-

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[2] All nutrients except fat and carbohydrate were supplied in a fixed relationship to calories. The following levels were supplied per 1000 Cal.: minerals (Hegsted et al., '41), 8 gm; choline, 1 gm ; inositol, 0.2 gm; vitamin A acetate, 3000 units; and crystalline calciferol, 400 units. Water soluble vitamins were supplied at the levels previously described by Mann et al. ('53).

ture tubes fitted with Teflon caps. The filtrates were stored in a nitrogen-filled desiccator at 0 to 5° until the analyses were made.

Tissue biopsies were obtained under light intravenous sodium amytal anesthesia. Approximately 40 mg of skin was obtained by making a small football-shaped incision on the dorsal surface of the calf of the leg. This section was completely devoid of any visible subcutaneous fat. A biopsy of similar size was taken from the gastrocnemius muscle. The biopsy area was repaired with inverting sutures of fine catgut. In certain instances a single testicle was removed through a small incision in the distal surface of the scrotum. Tissues were weighed, macerated with a Stadie blade, and finely ground with sand and a stirring rod in the bottom of a wide-mouth, glass-stoppered tube. Lipids were extracted and stored in a manner similar to that described for serum.

Analyses of lipids

Determinations were performed on either whole lipid extracts or on the cholesterol ester fraction eluted from silicic acid columns with light petroleum ether: chloroform (1:1 v/v) (Wycoff and Parsons, '57; Pinter et al., '58). Evaporation of the solvents from the whole lipid extracts and from the cholesterol ester fractions was carried out in a stream of nitrogen at 40°. After hydrolysis in 10% alcoholic KOH and addition of equal parts of water to the hydrolysis medium, the non-saponifiable and saponifiable fractions were extracted with ligroine. Cholesterol determinations were performed on the non-saponifiable fractions of the total lipid extract and of the column fraction by the method of Abell et al. ('52). Polyunsaturated fatty acid determinations were performed with the linoleic lipoxidase method of MacGee ('58) or with the microalkali isomerization technique of Holman and Hayes ('58). The determinations of total fatty acid concentrations were carried out by the titration method of Entenman ('57).

RESULTS

Depletion of polyunsaturated fatty acids from sera

Two monkeys which had been maintained on diets supplying 45% of calories as corn oil and two which had been fed 15% of calories as corn oil for 8 months were used in the first experiment. At zero time the 4 animals were changed to diets essentially devoid of fat and the monkeys were bled at intervals for determination of the concentrations of free and ester cholesterol, total fatty acids, total PFA, and PFA esterified with cholesterol.

The concentrations of PFA determined at time zero (fig. 1) are in the approximate range observed for these monkeys during the previous 8 months when they were fed either 15% or 45% of calories as corn oil. The level of PFA in the sera of all 4 monkeys had fallen after one day on the fat-free diet, and after 4 days the concentrations had reached levels which were only slightly higher than those seen later after 23 days on the fat-free diet. The levels of serum PFA expressed as a percentage of

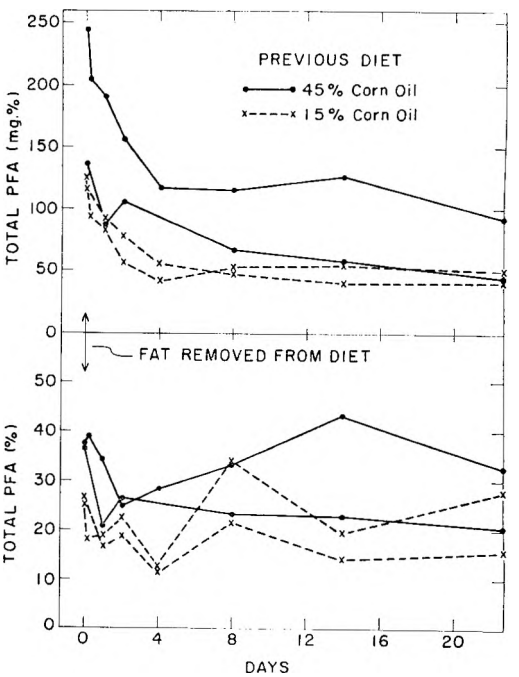

Fig. 1 The effect of changing from a diet containing corn oil to one essentially free of fat on the total polyunsaturated fatty acid (PFA) content of sera from Cebus monkeys. Two monkeys had been fed diets containing 15% of calories as corn oil and two 45% of calories as corn oil for 8 months. The upper part of the figure shows the serum PFA as mg per 100 ml, and the lower part presents the PFA as a percentage of total fatty acids.

the total serum fatty acids decreased only slightly during the period of study. This latter observation is a reflection of a decrease in the concentrations of saturated and monounsaturated fatty acids as well as that of PFA.

The decrease in PFA concentrations in the serum cholesterol ester fractions from these same monkeys is indicated in figure 2. The decrease, whether expressed as milligrams per cent or as the percentage of the fatty acids in the cholesterol ester fraction, was rapid and large. The concentrations of ester cholesterol (and ester cholesterol fatty acids) in the sera of the 4 monkeys were not changed significantly by the change from a corn oil-containing to a fat-free diet during the period of observation.

The levels of PFA measured in this experiment were determined exclusively with the linoleic lipoxidase method of MacGee ('58). A previous study (Portman et al., '59) had indicated that the predominant PFA in the serum of Cebus monkeys was dienoic (presumably linoleic acid). When fat-deficient diets were fed, the reduction in PFA was largely in the dienoic acids. The absolute levels of trienoic acids in the sera of fat-deficient animals were somewhat greater than those seen in control animals fed high levels of corn oil. Gas-liquid chromatographic analyses of serum lipids from Cebus monkeys fed corn oil also indicated that linoleic acid was the most prevalent PFA.

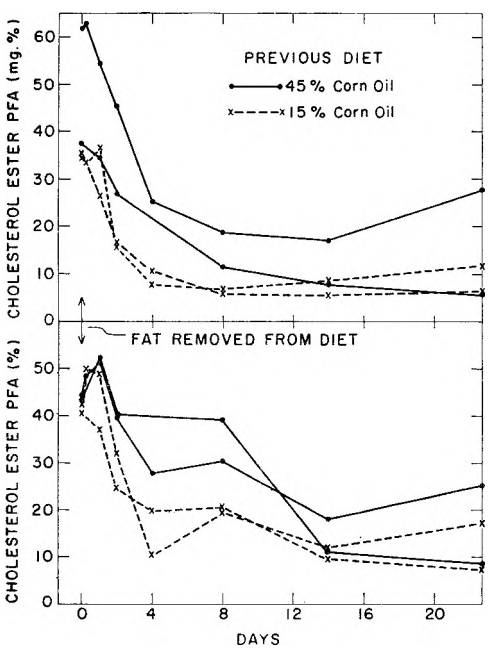

Fig. 2 The effect of changing from a diet containing corn oil to one essentially free of fat on the levels of polyunsaturated fatty acids esterified with cholesterol in the sera of Cebus monkeys. Conditions were as indicated for figure 1.

Repletion and disappearance of polyunsaturated fatty acids in sera

Six Cebus monkeys were fed diets essentially devoid of fat for periods of from 7 to 10 months. At time zero the monkeys were given a single daily ration of the high-corn oil-containing diet (45% of calories as fat). Since it had been determined, in a preliminary experiment, that the greater part of the deficit in serum PFA between animals fed high-corn oil and those fed fat-free diets was overcome after a single high-fat meal, it was decided to return the monkeys to a fat-free diet after this single corn oil meal. This provided a means for observing the rate at which the increase in PFA resulting from this single meal disappeared from the sera.

Figure 3 illustrates the time sequence in which the total serum PFA rose and declined in these monkeys. At 4 and 6 hours after the high-fat meal the serum PFA concentrations had risen to a mean figure of around 170 mg % compared to mean values of around 30 mg % for monkeys consuming a fat-free ration. The serum PFA content, 48 hours after consuming the high-fat meal, had decreased to about 70 mg% and decreased at a slower rate after that time. The total PFA content expressed as a percentage of the total fatty acids presented a similar pattern, although, as in the first experiment, there was also a drop in total fatty acids.

The concentration of PFA in the cholesterol ester fractions from sera of the monkeys given a single high-corn oil-containing meal, are shown in figure 4. The peak concentration in this fraction occurred after that seen for PFA in the total lipid fraction. The rate and extent of decrease in the PFA content of the cholesterol ester and in that of the total lipid fractions were almost identical.

Four more Cebus monkeys which had been fed diets containing 45% of calories as coconut oil[3] for 7 to 10 months, were also given a single meal containing 45% of calories as corn oil. On the following day the monkeys were returned to the coconut oil diet. The patterns of increase and fall-away in serum polyunsaturated fatty acids in the total lipid and the sterol ester fractions are indicated in figures 5 and 6. The base line (zero time) values for polyunsaturated fatty acids were considerably higher in this group fed coconut oil than in those of the previous study in which fat-free diets were fed. The polyunsaturated fatty acid concentrations in both the total lipid and cholesterol ester fractions rose sharply but not nearly to the same extent as in the monkeys fed fat-free diets in the preceding experiment. Six days after the single corn oil meal the polyunsaturated fatty acid concentrations in sera were still much higher than base line values.

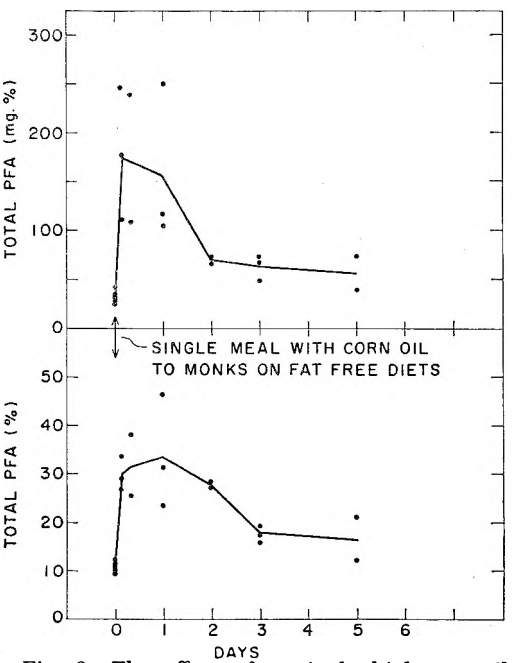

Fig. 3 The effect of a single high-corn oil (20 gm) meal on the total serum polyunsaturated fatty acid concentrations in monkeys that had been fed fat-free diets during the previous 8 months. The corn oil-containing meal was offered immediately after drawing blood at time zero. The monkeys were returned to the fat-free diet on day one.

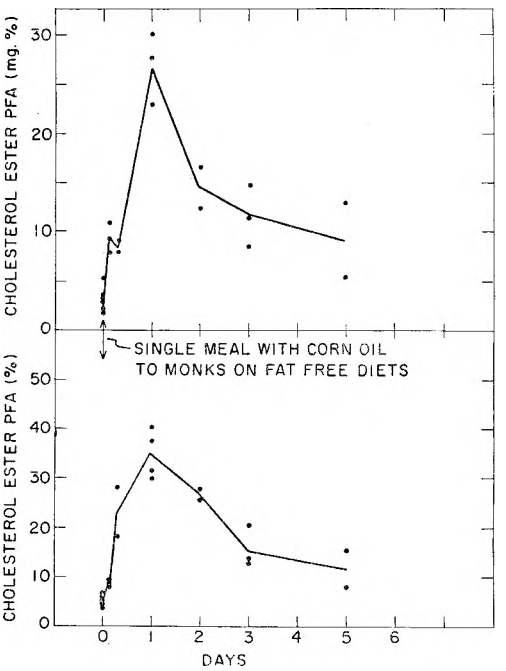

Fig. 4 The effect of a single high-corn oil meal on the polyunsaturated fatty acid concentrations in the cholesterol ester fractions of sera from Cebus monkeys which had been previously fed fat-free diets for 8 months. Conditions were as indicated in figure 3.

Rates of depletion and repletion of polyunsaturated fatty acids in tissues

Two Cebus monkeys which had been fed fat-free diets for 8 months were changed to diets supplying 45% of calories as corn oil at time zero, and two monkeys which had been on high-corn oil diets were changed to fat-free diets at the same time. Polyunsaturated fatty acid determinations by the alkali isomerization procedure were performed on the total lipid extract and on the sterol ester fraction from sera and from biopsies of skin, muscle, and testis. Two additional animals in each group were used for determinations on sera, skin, and muscle at time zero. Samples of sera and tissue were similarly analyzed at 10, 19, and 31 days after the diet changes.

[3] The mean value for the concentration of linoleic acid in the coconut oil, as determined by the alkali isomerization technique, was 3.5%. Linoleic acid was 6.1% of the identified fatty acids containing 10 or more carbon atoms, as determined by gas-liquid chromatography.

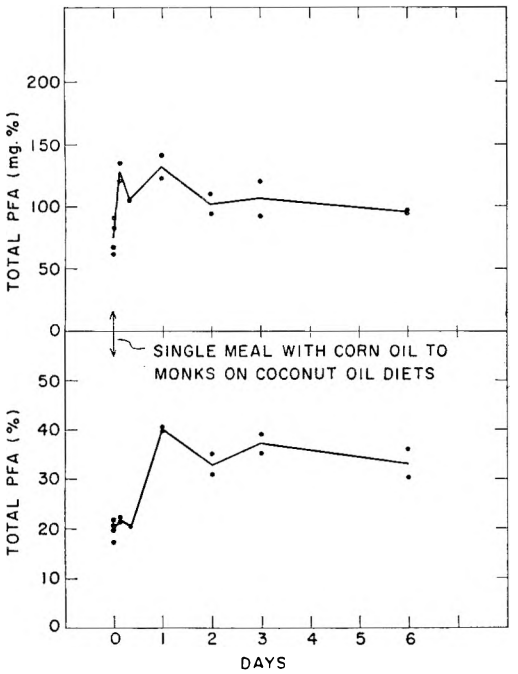

Fig. 5 The effect of a single high-corn oil meal on the total serum polyunsaturated fatty acid concentrations in monkeys that had been fed diets containing 45% of calories as coconut oil for 8 months. The monkeys were returned to the coconut oil diet at day one.

The concentrations of fatty acids of different degrees of polyunsaturation in the various tissues of monkeys which had been maintained for prolonged periods on fat-free and high-corn oil diets, are illustrated in table 1. The dienoic acids were abundant in all of the tissues analyzed from the corn oil-fed monkeys. In the single testis of the animals from this group the tetraenoic acids appeared to predominate. The level of dienoic acids was markedly lower in the tissues of monkeys fed fat-deficient diets, and the ratio of dienoic to trienoic acids was reduced. It is interesting that the dienoic acids comprised a very large proportion of the total PFA esterified with cholesterol in the sera. The greatest differences in the total PFA concentrations between the corn oil and the fat-free monkeys occurred in the skin.

The changes in tissue lipid analyses which occurred when diets were changed from fat-free to high-corn oil and vice versa are indicated in figure 7. The figure for total PFA is the sum of the fatty acids determined by the alkali isomerization technique to contain two or more double bonds. Ten days after the reversal of diets the total PFA level in the serum of the monkeys, which were changed to a high-corn oil diet, had reached the high levels existing in other monkeys fed this diet for several months. The levels in the sera of the monkeys changed from high-corn oil to fat-free diets had decreased slightly. At the 10-day interval the PFA concentrations in the cholesterol ester fractions had completely reversed, and levels were observed which were equivalent to those seen when the diets had been fed for prolonged periods. It is probable, on the basis of the previous experiments, that the monkeys changed to corn oil diets had already reached steady state values for serum PFA in less than 10 days. The reversal of PFA concentrations in muscle, skin, and testis was much slower; however, the reversal in PFA concentrations had oc-

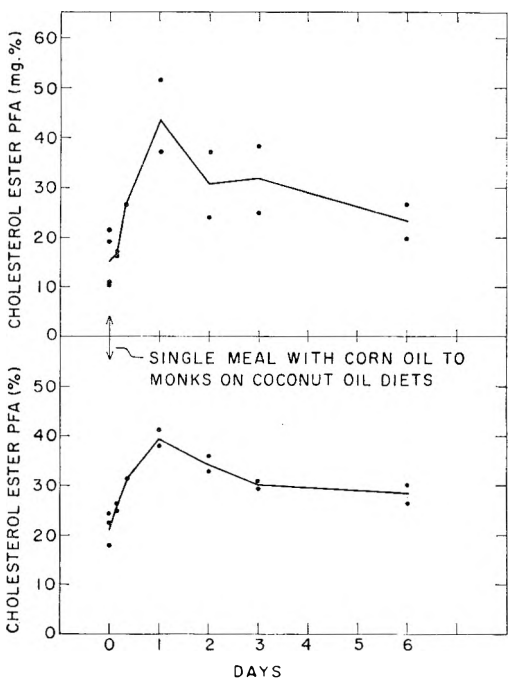

Fig. 6 The effect of a single high-corn oil meal on the polyunsaturated fatty acid concentrations in the cholesterol ester fractions of sera from Cebus monkeys which had been previously fed diets containing 45% of calories as coconut oil for 8 months. The monkeys were returned to the coconut oil diet at day one.

TABLE 1

The effect of feeding diets containing no fat or 45% of calories as corn oil on the polyunsaturated fatty acid distribution in the lipids obtained from various tissue biopsies of Cebus monkeys, fed the experimental diets for 8 months

(4 animals per group)

		Fatty acid concentrations					
		Total fatty acids	Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
		mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
Muscle	Fat-free	1,232.0	142.0	91.9	78.8	16.3	22.0
	45% corn oil	1,848.0	536.0	38.6	106.2	23.9	49.2
Skin	Fat-free	4,420.0	29.8	40.6	39.8	6.6	0.8
	45% corn oil	13,250.0	5,480.0	115.2	193.3	109.4	195.9
Testis	Fat-free	3,080.0	148.7	348.5	773.9	146.1	78.1
	45% corn oil	3,500.0	863.3	47.0	1,002.1	178.3	396.9
Serum	Fat-free	277.0	19.3	25.8	9.4	2.2	5.4
	45% corn oil	344.0	148.0	5.8	32.8	2.6	6.8
Serum cholesterol ester	Fat-free	84.9	2.8	1.6	1.1	0.2	2.0
	45% corn oil	126.1	41.9	2.5	3.1	0.6	2.7

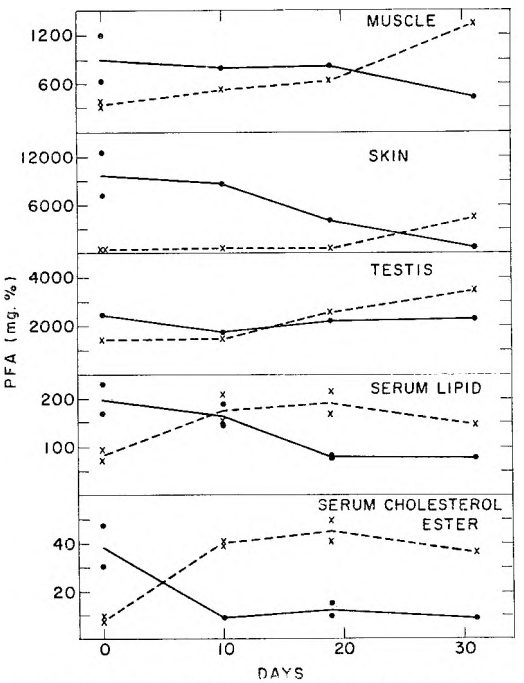

Fig. 7 The effect of changing the diet from one supplying 45% of calories as corn oil to one devoid of fat, and vice versa, on the total polyunsaturated fatty acid concentrations in tissue lipids of Cebus monkeys. The monkeys had been fed diets for 8 months prior to reversal of diets at time zero. X—X indicates the monkeys changed from a fat-free diet to the high corn oil diet; ●—● is the reverse change.

curred in these tissues by 31 days after the diet reversal.

The ratio of dienoic to trienoic acids in the fatty acid fractions was also highly dependent on the diet fed. The changes which occurred in this ratio when the diets were reversed are indicated in figure 8. The change of dienoic/trienoic acid ratios to those characteristic of the steady state values observed when the new diets were fed for prolonged periods, occurred most rapidly in the total serum fatty acids and in the serum cholesterol ester fatty acids. Values for the muscle, skin, and testicular lipid changed more slowly.

DISCUSSION

These studies indicate that the PFA content of the total lipid of serum and of the serum cholesterol ester fraction from Cebus monkeys are rapidly influenced by the fatty acid composition of the dietary fat. The exchange of PFA from fat depots, as represented by skin, muscle, and testicular lipid, with the serum fatty acids is, on the other hand, slow. Monkeys which had been fed fat-free diets for 8 months, and in which the PFA levels in sera and tissues were low, had their serum PFA levels restored essentially to the steady-state values of monkeys fed a high-corn oil diet for long periods when they were given a single meal rich in corn oil. Furthermore, the rate of decrease of PFA from the sera of the monkeys given the single corn oil meal was scarcely more rapid than that observed in monkeys which had been fed corn oil containing diets for 8 months before the institution of a fat-free diet. The PFA content of tissues from monkeys fed corn oil and those fed fat-free diets for long periods were strikingly different, but the rate of change in tissue composition was slow compared to that of sera when the diets were reversed. Thus, a large proportion of the PFA in the sera seemed to be derived directly from the diet and to be only slowly exchangeable with the tissue fat.

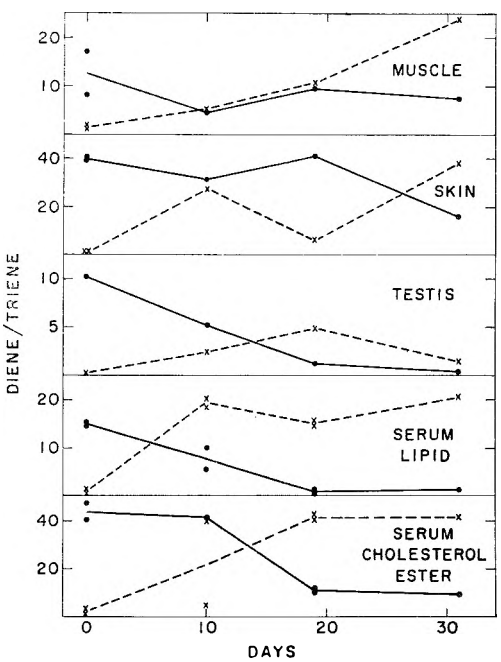

Fig. 8 The effect of changing the diet from one supplying 45% of calories as corn oil to one devoid of fat, and vice versa, on the ratio of dienoic to trienoic acids in tissue lipids of Cebus monkeys. Legend is otherwise that of figure 7.

There was little indication that the decreases in the PFA of the cholesterol ester fraction which occurred after monkeys given a single corn oil meal were returned to a fat-free diet, were different in rate or extent from the changes seen in the total lipid fraction. When monkeys previously maintained on a fat-free diet were given a corn oil meal, the PFA concentration of the cholesterol ester fraction rose more slowly than the PFA of the total lipid fraction. This observation is compatible with the finding that esterified cholesterol absorbed from the gut appears slowly in the chyle and peripheral circulation (Swell et al., '58). The data do not allow one to decide whether the PFA in the cholesterol ester fraction were esterified with cholesterol in the gut mucosa or at other sites. The apparently similar rates of decrease of PFA concentrations from the cholesterol ester and total lipid fractions, and the similar composition of these fractions (expressed as percentages of the fatty acids which were polyunsaturated) suggest that the total serum lipid PFA and the cholesterol ester PFA are metabolized at similar rates. This observation may be related either to equal rates of metabolism of the various chemical subclasses of lipids or to rapid exchange of the PFA between classes.

SUMMARY

The pattern of depletion and repletion of polyunsaturated fatty acids (PFA) in the serum and certain tissues from Cebus monkeys, which were changed from diets very rich in linoleic acid to fat-free diets and vice versa, were studied. When monkeys fed diets containing 45 or 15% of calories as corn oil for 8 months were changed to fat-free diets, the total PFA concentrations as well as the total fatty acid levels in sera declined rapidly. The polyunsaturated fatty acid concentrations in the serum cholesterol ester fractions also declined sharply and were largely replaced by monounsaturated and saturated fatty acids. Monkeys that had been fed fat-free diets or coconut oil diets for 8 months had their deficit of PFA in the serum total lipid and cholesterol ester fractions corrected after a single meal containing corn oil. The elevations above base line values persisted for several days. The PFA concentrations and the ratios of dienoic to trienoic acids in the lipids of skin, muscle, and testis changed much more slowly than did the values for serum lipids when the diets were changed from high-corn oil diets to fat-free diets, and vice versa.

LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

Ahrens, E. H., Jr. 1957 Nutritional factors and serum lipid levels. Am. J. Med., *23:* 928.

Deuel, H. J., Jr. 1957 The Lipids; Their Chemistry and Biochemistry. Interscience Publ., New York, vol. 3, p. 783.

Entenman, C. 1957 Methods in Enzymology. Edited by S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 299.

Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

Holman, R. T., and H. Hayes 1958 Determination of polyunsaturated acids in lipides of plasma and tissue. Anal. Chem., *30:* 1422.

MacGee, J. 1958 Enzymatic determination of polyunsaturated fatty acids. Ibid., *31:* 298.

Mann, G. V., S. B. Andrus, A. M. McNally and F. J. Stare 1953 Experimental atherosclerosis in Cebus monkeys. J. Exp. Med., *98:* 195.

Pinter, K., O. W. Portman and D. M. Hegsted 1958 Dietary fat and hypercholesteremia in the Cebus monkey: Polyunsaturated fatty acids in subfractions of serum lipides. Federation Proc., *17:* 488.

Portman, O. W., D. M. Hegsted, F. J. Stare, D. Bruno, R. Murphy and L. Sinisterra 1956 Effect of the level and type of dietary fat on the metabolism of cholesterol and beta lipoproteins in the Cebus monkey. J. Exp. Med., *104:* 817.

Portman, O. W., K. Pinter and T. Hayashida 1959 Dietary fat and hypercholesteremia in the Cebus monkey. III. Serum polyunsaturated fatty acids. Am. J. Clin. Nutrition, *7:* 63.

Swell, L., E. C. Trout, Jr., J. R. Hopper, H. Field, Jr. and C. R. Treadwell 1958 Mechanism of cholesterol absorption. II. Changes in free and esterified cholesterol pools of mucosa after feeding cholesterol-4-C^{14}. J. Biol. Chem., *233:* 48.

Wycoff, H. D., and J. Parsons 1957 Chromatographic microassay for cholesterol and cholesterol esters. Science, *125:* 347.

Fatty Acid Distribution in Egg Yolk as Influenced by Type and Level of Dietary Fat

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The proportions of the various lipid fractions of egg yolk appear to be unaltered by the fat content of the diet according to the results of Reiser ('50), in which the moisture, total lipid, phospholipid, or cholesterol content was not different in eggs from hens on either a "fat-free" diet or one containing 4% of cottonseed oil.

It appears to be well established, however, that the distribution of fatty acids in egg yolk varies considerably with the dietary fat. Somewhat variable results are therefore to be expected in analyses for fatty acid distribution in egg yolk. The detailed analysis of fatty acid composition of egg yolk reported by Riemenschneider et al. ('38) differs somewhat from that reported by Shorland ('51). While differences in analytical methods may account in part for such differences, it is also quite likely that the diet of the hens may be a factor. The egg yolks analyzed by Shorland were from hens of unknown dietary history, whereas those analyzed by Riemenschneider et al. were from hens fed diets containing 2% of cod-liver oil and 7% of fishmeal, which may account for the relatively high level of C-22 unsaturated fatty acids found by the latter group.

The unsaturated fatty acid portion of the egg yolk, in particular, is the fraction most susceptible to dietary manipulation. This was shown by Cruickshank ('34), employing calculations based on the determination of solid acids, iodine values and thiocyanogen values. She reported variations in fatty acids of egg yolks from hens fed high levels (28%) of various fats. Thus, on feeding a highly saturated fat, palm kernel oil, she reported 16.1% of the total fatty acids as linoleic acid, but on feeding a highly unsaturated fat, hemp oil, the linoleic acid content of the yolk was found to be 41.7%. The linolenic acid content of the yolk could be increased 10-fold by feeding linseed oil. Reiser ('51), feeding hens various fats at low levels (2%), showed that either a fat-free diet or the presence in the diet of a highly saturated fat (bayberry tallow) would result in reduction of the dienoic acid content of the yolk to very low levels. The polyunsaturated fatty acid fraction could be considerably increased, even at these low dietary fat levels, by feeding, for example, tung oil or cod-liver oil. Increases in yolk levels of polyunsaturated fatty acids are accompanied by corresponding decreases in "oleic" acid (Cruickshank, '34; Reiser, '51; Feigenbaum and Fisher, '59), but "oleic" acid levels are not influenced by feeding the highly saturated fats. Fisher and Leveille ('57) studied egg yolk levels of two fatty acids, linoleic and linolenic, on feeding 20% of fat in the diet. The yolk content of these fatty acids was proportional to the levels in the diet. A more recent study by Feigenbaum and Fisher ('59) indicates that when various oils were fed at 10% of the diet the composition of the egg yolk fat was influenced only by the dietary polyunsaturated fatty acids whereas the composition of the body fat was influenced by either saturated or unsaturated fatty acids in the diet.

The present investigation was carried out for two reasons: (1) no report has thus far appeared in which a study employing specific analytical techniques has been made of the effect of dietary fat upon all the major fatty acid components of egg yolk, and (2) the apparent nutritional importance of linoleic acid makes it desirable to define conditions under which its content in foods can be varied.

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METHODS

1. Diets. Single-comb White Leghorn hens were fed the diets shown in table 1. Added fat provided from 6.4 to 69% of the total Calories. The energy content of the ration was calculated using average values for metabolizable energy from the tables of Hill and Renner ('57). To enhance the stability of the fat, fortification with tocopheryl acetate was provided as indicated. The safflower oil employed had been stabilized with propyl gallate (0.01%) and 0.01% of citric acid. Also, a purified wood cellulose,[1] was included in some of the diets, not because the oily diets were unacceptable to the hens, but in order to facilitate handling by decreasing their oiliness. The experiments of Williams and Grau ('56) indicate that cellulose may be considered nutritionally inert for young chicks, and this is assumed here to hold for hens also. Thus in the latter half of the experiment the diets containing 22.2 and 30% of oil were diluted by adding 5 parts of cellulose to 95 parts of diet. In the tables these diets are still referred to as 22.2 and 30% oil for convenience since the fundamental relationship is not the percentage of oil in the diet but the ratio of energy supplied by the oil to that of the total diet, and this was not changed by

[1] Solka Floc SW40A, Mefford Chemical Co., Los Angeles 22, California.

TABLE 1

Composition of experimental diets

Ingredients	Basal ration	Stock ration
	Parts per 50	%
Ground corn	—	14.20
Ground barley	—	45.00
Ground milo	—	10.00
Soybean meal (44%)	34.00	3.00
Fishmeal	—	4.375
Wheat bran	1.325	7.50
Alfalfa meal	5.00	5.00
Dried whey	2.00	2.50
Dried skim milk	—	3.75
Limestone	2.00	3.00
Steamed bone meal	4.00	1.00
Iodized salt	0.500	0.25
Manganese sulfate	0.025	0.125
Feeding oil (2250A–300D)	—	0.25
Vitamins[1]	—	0.05
Vitamin mix[2]	1.150	—
Totals	50.00	100.00

[1] Fortafeed 2–22, American Cyanamid Company, New York, N. Y. Contains riboflavin, pantothenic acid and niacin, each 2 gm per pound.

[2] Contains in grams: vit. A (10,000 I.U./gm) 100.00, vit. D (1000 U/gm) 66.0, tocopheryl acetate (44 U/gm) 100.00, vitamin B_{12} (1 mg/gm) 0.30, riboflavin 0.40, Ca pantothenate 0.25, niacin 0.80, choline chloride (25%) 40.00, butylated hydroxytoluene 11.50, sucrose 827.75.

	Experimental rations					
Basal	50.0	40.0	43.0	49.0	55.5	60.0
Ground barley	—	34.0	47.0	36.0	22.3	—
Pearl cornstarch	48.0	17.5	—	—	—	—
Cellulose	—	—	—	—	—[1]	—[1]
Wheat bran	—	—	—	—	—	10.00
Vegetable oil	2.0[2]	5.0[3]	10.0[2,3,4]	15.0[3]	22.2[3,5]	30.0[3,5]
% Calories from added fat	6.4	16.0	29.3	40.8	54.5	69.0

[1] Diet diluted by adding 5 parts Solka Floc to 95 parts diet in latter half of experiment (see text).

[2] Soybean oil.

[3] Safflower oil.

[4] Corn oil, or cotton seed oil.

[5] Linseed oil.

this manipulation. The chicken eats food primarily to supply its requirement for energy (Hill and Dansky, '54; Peterson et al., '54; Williams and Grau, '56). For this reason the diets were designed to maintain a constant relationship between energy and protein levels. Fresh diets were made up every second day and stored in brown bottles in the refrigerator. Remaining feed was discarded each morning and fresh feed added. In some cases hens stopped laying completely or temporarily. Eggs used for analysis were obtained only from those hens which laid continuously throughout the experiment.

2. *Analytical.* Eggs were kept under refrigeration until they could be broken out and separated; then the yolks were stored at −20°C until they could be analyzed. Those from the hens fed 22.2 and 30% levels of oil, the stock ration, and the 2% oil ration were analyzed individually. With the groups of hens fed 5, 10, and 15% levels of oil, yolks of eggs from the same time interval on the same diet were pooled. Samples of approximately 600 mg were extracted repeatedly with boiling ethanol-acetone (1:1, v/v). The extracts were filtered and made up to a volume of 200 ml. This gave an approximate concentration of 1 mg of lipid per milliliter of filtrate. Aliquots of this acetone-alcohol filtrate were taken for the determination of total lipid by Bragdon's ('51) method, for free and total cholesterol and for iodine value by the method of Michaels et al. ('58), and for lipid phosphorus by the method of Fiske and Subba-Row ('25). Polyunsaturated fatty acids were determined on the series of yolk extracts from the high-fat diets by the alkaline isomerization method of Holman ('57) as modified by Michaels ('58). Extracts of representative eggs from this group were also methylated by diazomethane, after saponification with sodium ethylate, and the methyl esters subjected to gas-liquid chromatography. The instrument used was an Aerograph-110C with a column of LAC-2R-446 using helium as the carrier gas. Alkaline isomerization was not done on the pooled egg yolks obtained on the lower fat diets, but aliquots of the extracts were methylated by refluxing for one hour with 10 ml of 1% sulfuric acid in absolute methanol and analyzed by gas-liquid chromatography. In this case the column used was packed with "Craig's polyester" (Murty and Craig, '58), a polymer of butanediol and succinic acid, and helium was again the carrier gas. Retention volumes with this column are very

TABLE 2

Composition of oils fed hens as determined by gas-liquid chromatography

Oil	Fatty acid composition							
	Myristic	n-C_{15}?	Palmitic	Palmitoleic?	Stearic	Oleic	Linoleic	Linolenic
	%	%	%	%	%	%	%	%
Safflower no. 1			6.6		3.8	12.7	76.9	
Safflower no. 2			8.4		4.2	13.7	73.8	
Linseed	0.4	0.3	5.9		2.4	17.2	15.5	58.3
Cottonseed	0.7		22.1	1.3	3.2	15.6	57.1	
Corn			10.7		3.2	28.4	54.7	3.0
Soybean			10.1		5.0	21.6	54.5	8.8

TABLE 3

Effect of high-fat diet on lipid fractions of eggs

Diet of hens	No. of eggs	Total lipid	Total cholesterol	Phospholipid
		%	%	%
Stock—low-fat	16	32.35 ± 0.33[1]	1.54 ± 0.04	9.56 ± 0.13
20% and 30% fat, 4–6 weeks	12	32.32 ± 0.36	1.44 ± 0.05	10.21 ± 0.14[2]

[1] Standard error of the mean.
[2] Difference significant at the 1% level.

similar to those with LAC-2R-446, so that chromatograms from the two columns can be compared. Methyl esters prepared from the oils fed the hens were also run on the gas column. The composition of these oils is shown in table 2.

RESULTS AND DISCUSSION

Our results extend those of Reiser ('50) in that the total lipid and free and esterified cholesterol contents of the eggs from hens fed up to 30% of oil in the diets did not differ significantly from the values obtained with eggs from hens on the stock diet or on the control diet. However, the values for phospholipids were significantly higher on the high-fat diets. These data are shown in table 3. Eggs produced elsewhere on a 20% safflower oil diet for use in a metabolic study were found to average 1.8 gm heavier than those from hens on a stock diet. The samples included 1350 eggs from each group.[2] The flavor of the "safflower" eggs did not differ from that of the control eggs, but eggs from hens fed high linseed oil diets were reported to have off flavors by a taste panel at the Western Utilization Research and Development Division at Albany, California.[3] This is in agreement with results previously found by Cruickshank ('34).

Results of alkaline isomerization analysis of the series of eggs from hens fed 22.2 and 30% fat are shown in table 4. Values obtained by gas-liquid chromatography of methyl esters from extracts of some of these eggs and of the eggs from hens fed lower levels of oils, are shown in table 5. Figure 1 shows the relationship between the level of linoleic acid in the diet of the hens and that in the eggs, as determined by gas-liquid chromatography. From the results in figure 1 and tables 4 and 5 it would appear that the linolenic acid in the dietary fat interferes with the incorporation of linoleic acid into the egg fat. Eggs from hens fed the 30% linseed oil diet (4.4% linoleic acid in the diet)

[2] Lamoreux, W. F. 1958 Personal communication.

[3] Lineweaver, H. 1958 Personal communication.

TABLE 4

Fatty acids of total lipids from egg yolk as determined by alkaline isomerization

Diet of hens	Weeks on diet	No. of eggs	Iodine number	Fatty acids, %				
				Saturated	Monoenoic	Dienoic	Trienoic	Tetraenoic
Stock		19	75	35.2	51.8	8.4	1.7	1.3
2% Soybean oil (control)		9	72	37.0	51.5	7.8	1.1	1.3
22.2% Safflower oil	1	2	83	34.1	39.1	20.6	1.0	1.3
	2	2	88	42.5	24.9	28.6	0.6	1.7
	3	2	86	45.4	20.6	29.8	1.3	2.0
	4	2	86	40.0	29.4	27.4	0.8	1.4
	6	1	92	41.6	23.1	31.1	1.7	1.6
30% Safflower oil	1	1	91	40.6	26.9	26.3	3.3	2.1
	2	1	96	37.2	25.5	33.1	1.8	2.2
	3	1	92	45.6	15.8	33.2	1.6	2.5
	4	1	93	43.3	17.2	35.2	1.3	2.0
	6	2	94	41.5	20.0	33.8	2.2	1.8
22.2 % Linseed oil	1	1	107	34.6	34.4	12.6	15.3	1.4
	2	2	102	39.4	30.2	12.5	14.6	1.4
	3	1	94	39.2	34.8	11.0	12.8	1.1
	4	2	98	36.1	36.8	12.0	12.5	1.3
	6	2	99	40.7	30.9	11.5	15.3	1.2
30% Linseed oil	1	2	97	36.5	37.9	10.3	12.2	1.2
	2	1	93	43.1	30.0	11.0	12.8	1.2
	3	1	94	36.8	39.7	8.9	11.9	1.2
	4	1	95	39.6	34.6	9.3	14.4	1.2
	5	1	96	39.4	33.9	10.0	14.1	1.4

TABLE 5

Fatty acids of total lipids from egg yolk as determined by gas-liquid partition chromatography

Diet of hens	Weeks on diet	Fatty acids								
		Myristic	Palmitic	Palmit-oleic?	C_{17} n-sat.?	Stearic	Oleic	Lino-leic	Lino-lenic	Arach-idonic
		%	%	%	%	%	%	%	%	%
Stock	"Prediet"	0.6+	26.5	8.1		8.1	46.8	9.6		
Stock	1	0.4	26.6	5.3		11.7	46.5	9.5		
Stock	6	0.5	28.6	6.1		11.1	47.8	6.0		
2% Soybean oil	6	0.6	24.5	5.2		10.1	46.6	13.0		
5% Safflower oil	3	0.2	26.4	2.2		13.3	35.7	22.2		
5% Safflower oil	5	0.3	24.2	2.4	0.3	13.6	34.0	23.1		2.2
10% Safflower oil	3	0.3	23.7	2.2	0.4	13.3	29.4	28.0		2.7
10% Safflower oil	5	0.4	23.6	1.9	0.7	11.9	28.6	32.9		
15% Safflower oil	3		24.2	2.2		8.5	31.7	33.4		
15% Safflower oil	5	0.4	26.4	2.0	0.7	12.2	27.0	31.4		
22.2% Safflower oil	3	0.2	23.4	3.1		11.1	27.2	35.0		
22.2% Safflower oil	4	0.2	25.2	2.0	0.8	11.5	24.6	35.7		
22.2% Safflower oil	6	0.2	24.8	1.2		11.9	23.5	35.6	2.9	
30% Safflower oil	2	1.4	22.9	not sep.	1.0	11.3	22.7	40.7		
30% Safflower oil	4	0.2	24.6	not sep.		10.8	22.6	41.8		
30% Safflower oil	6		22.3	not sep.		10.9	22.9	41.1	2.8	
10% Cottonseed oil	3	0.4	28.3	1.8	0.6	14.9	26.7	23.8		3.6
10% Corn oil	3		22.1	2.3	0.6	14.3	38.9	21.8		
10% Soybean oil	3	0.3	20.8	2.8	0.6	12.4	39.9	19.3	2.2	1.9
22.2% Linseed oil	6	0.3	20.2	3.1		11.0	35.8	14.1	14.0	1.5
30% Linseed oil	4	0.3	17.4	3.0		13.8	40.2	11.9	13.5	
30% Linseed oil	5	0.4	16.1	3.2		16.1	40.3	13.3	10.5	

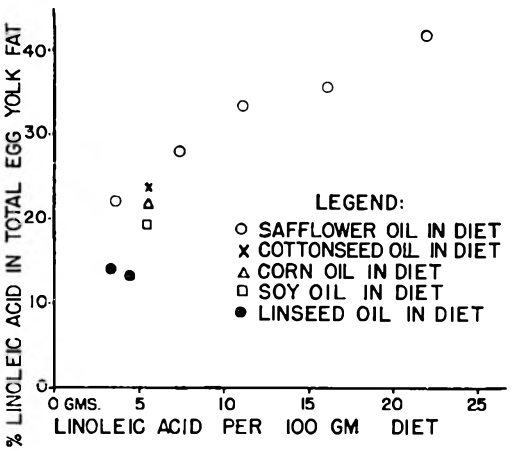

Fig. 1 Relationship between percentage of linoleic acid in total fatty acids of egg yolk fat and percentage of linoleic acid in diets fed hens.

contained only slightly more than half as much linoleate as those from hens consuming the 5% safflower oil diet (3.7% linoleic acid in the diet). This result is not due merely to equivalent replacement of linoleic acid by linolenic acid since if the sums of values for linolenic and linoleic acids are plotted as in figure 1, the points still fall considerably below the curve for safflower oil (and cottonseed oil). The points for soybean oil and corn oil, both of which contained linolenic acid, fall somewhat below the curve for safflower oil, while the point for cottonseed oil, containing no linolenic acid, falls on the curve. Three lots of corn oil (including the one fed the hens) were analyzed by gas-liquid chromatography and found to contain three to 4% of a fatty acid whose methyl ester had the same retention volume as methyl linolenate, and which disappeared after bromination. This is at variance with literature values which indicate only traces of linolenic acid in corn oil.

Increases in linoleic acid in the eggs were accompanied by corresponding decreases in oleic and palmitoleic acids. The levels of saturated fatty acids did not decrease except in the instances where linoleic acid was about 40% of the total fatty acids, when there was a slight decrease in the level of palmitic acid (tables 4 and 5). The inverse relationship between the percentage of linoleic acid and that of oleic acid in the eggs is shown in figure 2. In the eggs from hens fed linseed oil there appeared to be a decrease in levels of palmitic acid as well as in the monoenoic acids as the levels of linoleic and linolenic acids in the eggs increased. The change in composition of yolk fatty acids with dietary fat is most strikingly illustrated in figure 3 which shows tracings of chromatograms of methyl esters of fatty acids from a control egg, an egg from a hen fed 30% of safflower oil, and an egg from a hen fed 30% of linseed oil.

The results for linoleic acid reported here by the gas chromatography method are higher than those reported in the equivalent experiment of Feigenbaum and Fisher ('59) on feeding 10% of safflower oil. These investigators employed an alkaline isomerization method for analysis of polyunsaturated fatty acids. A comparison of tables 4 and 5 shows that the values for linoleic acid and linolenic acids are lower by the alkaline isomerization method than by the more accurate gas-liquid chromatography method.

Neither analysis by gas-liquid chromatography nor alkaline isomerization showed any increase in arachidonic acid in the whole yolk fat. Values for pentaenoic and hexaenoic acids by alkaline isomerization were very low and variable, so are not considered to have any significance. No peaks that could have been these acids were observed on the chromatograms. This is

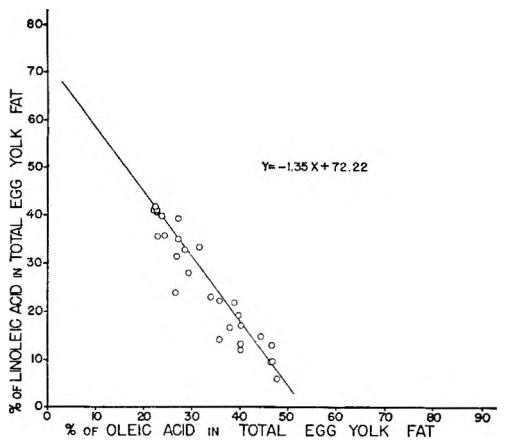

Fig. 2 Relationship between percentages of linoleic and oleic acids in total fatty acids of yolk fat in eggs of hens fed diets with varying contents of vegetable oils.

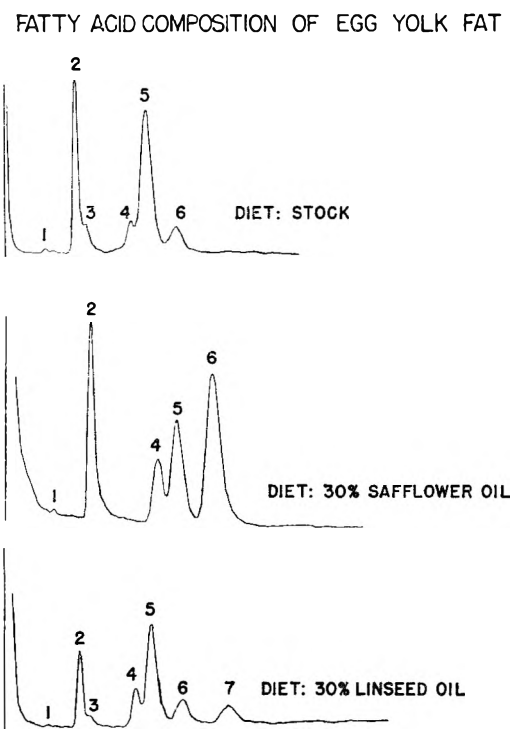

Fig. 3 Gas-liquid chromatograms of methyl esters of fatty acids from egg yolk fat. Diets of hens as indicated. Peaks are as follows: 1, myristate; 2, palmitate; 3, probably palmitoleate; 4, stearate; 5, oleate; 6, linoleate; and 7, linolenate.

probably to be expected if the isomerization values are correct, because peaks containing 1% or less of the total fatty acids would probably be too flat to be recognizable at the very large retention volumes to be expected for the methyl esters of C_{22} fatty acids with 5 or 6 double bonds. Under our experimental conditions, it is very likely that values for arachidonic acid are too low, because of the relative insensitivity of the detector to esters with a longer retention time than that of methyl linolenate. Palmitoleic acid was not identified by recovery from the column and degradation, but a peak was tentatively identified as that of methyl palmitoleate because (a) the relationship of the peak to that of methyl palmitate was the same as that of methyl oleate to methyl stearate, (b) on the log plot of retention volumes it fell in the C_{16} position on the line with methyl oleate and (c) it disappeared after bromination. In a few cases a small peak appeared between the "palmitoleic" and stearic acid peaks. This was tentatively identified as a n-C_{17} saturated fatty acid, from the plot of retention volumes. It does not disappear on bromination.

Eggs from hens fed the high linseed oil diets were comparable in linoleic acid content to those reported by Fisher and Leveille ('57). It is doubtful, however, that any real increase in linoleic acid occurred in these eggs. The values reported are higher than the *average* values obtained with the stock diets, but when records of individual hens were examined, it was found that the "prediet" levels of dienoic acid of eggs from hens later put on the linseed oil diets were higher than the average, and that increases in linoleate with the linseed oil rations were only 2 to 3%. The high levels of linoleic acid obtained by Cruickshank ('34) may have been referable to the unusually high (39%) linoleate content of the linseed oil used by her, while the linseed oil used in the present experiments had an unusually low linoleate content (15.5%). Linolenic acid, however, rose in these same eggs from zero to 2% on the stock diet to 14 to 16% in 6 weeks. These values are similar to those reported by Cruickshank and by Fisher and Leveille.

SUMMARY

Hens were fed diets containing 5, 10, 15, 22.2 or 30% of safflower oil; 22.2 or 30% of linseed oil; and 10% of cottonseed, corn, or soybean oil. The total fatty acids of the yolk fat were determined by alkaline isomerization and by gas-liquid chromatography and their distribution compared with that of eggs produced on a stock diet or on a low-fat diet.

The linoleic acid content of eggs from hens fed safflower oil or cottonseed oil was approximately proportional to the level of linoleic acid in the diet.

In eggs from hens fed diets containing linolenic acid (corn oil, soybean oil, linseed oil) linoleic acid was not deposited as efficiently as in those from hens fed safflower oil or cottonseed oil, suggesting an antagonistic or inhibitory effect of linolenic acid toward incorporation of linoleic acid into yolk fat.

On all diets, except the linseed oil diets, the principal change accompanying an increase in linoleic acid was a corresponding decrease in oleic and palmitoleic acids. Only at about 40% of linoleic acid was there a slight decrease in palmitic acid level. No change occurred in the level of stearic acid.

On the linseed oil diets the increases in yolk levels of polyunsaturated fatty acids were accompanied by decreases, not only in the monoenoic acids but also of palmitic acid, even though stearic acid remained unchanged.

Levels of tetraenoic, pentaenoic and hexaenoic acids were low and variable on all diets. No changes that could be ascribed to the diet used were found in these acids.

ACKNOWLEDGMENTS

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LITERATURE CITED

Bragdon, J. H. 1951 Colorimetric determination of blood lipides. J. Biol. Chem., *190:* 513.

Cruickshank, E. M. 1934 Studies in fat metabolism in the fowl. I. The composition of the egg fat and depot fat of the fowl as affected by the ingestion of large amounts of different fats. Biochem. J., *28:* 965.

Feigenbaum, A. S., and H. Fisher 1959 The influence of dietary fat on the incorporation of fatty acids into body and egg fat of the hen. Arch. Biochem. Biophys., *79:* 302.

Fisher, H., and G. A. Leveille 1957 Observations on the cholesterol, linoleic and linolenic acid content of eggs as influenced by dietary fats. J. Nutrition, *63:* 119.

Fiske, C. H., and Y. SubbaRow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

Hill, F. W., and L. M. Dansky 1954 Studies of the energy requirements of chickens. Poultry Sci., *33:* 112.

Hill, F. W., and R. Renner 1957 Metabolizable energy values of feedstuffs for poultry and their use in formulation of rations. Cornell Nutrition Conference for Feed Manufacturers, Proceedings, p. 22.

Holman, R. T. 1957 Measurement of polyunsaturated fatty acids. Methods Biochem. Anal., *4:* 99.

Michaels, G. D. 1958 A note on increasing the accuracy of the micro alkaline isomerisation technique for determination of unsaturated fatty acids. Am. J. Clin. Nutrition, *6:* 593.

Michaels, G. D., G. Fukayama, H. P. Chin and P. Wheeler 1958 Techniques for separation of plasma cholesterol esters, for determination of iodine value, and of cholesterol. Proc. Soc. Exp. Biol. Med., *98:* 826.

Michaels, G. D., P. Wheeler, G. Fukayama and L. W. Kinsell 1959 Plasma cholesterol fatty acids in human subjects as determined by alkaline isomerisation and by gas chromatography. Ann. N. Y. Acad. Sci., *72:* 633.

Murty, N. L., and B. M. Craig 1958 Fatty acid analysis of vegetable oils by gas-liquid phase chromatography. Reported at Chicago, October 21, 1958, at meeting of American Oil Chemists' Society.

Peterson, D. W., C. R. Grau and N. F. Peek 1954 Growth and food consumption in relation to dietary levels of protein and fibrous bulk. J. Nutrition, *52:* 241.

Reiser, R. 1950 Fatty acid changes in egg yolk of hens on a fat-free and a cottonseed oil ration. Ibid., *40:* 429.

——— 1951 The synthesis and interconversions of polyunsaturated fatty acids by the laying hen. Ibid., *44:* 159.

Riemenschneider, R. W., N. R. Ellis, and H. W. Titus 1938 The fat acids in the lecithin and glyceride fractions of egg yolk. J. Biol. Chem., *126:* 255.

Shorland, F. B. 1951 The fatty acid composition of egg yolk lipids. New Zealand J. Sci. and Technology, *33B:* 224.

Williams, M. A., and C. R. Grau 1956 Energy intake and body composition of the chick in relation to the dietary concentrations of digestible carbohydrate and digestible food energy. J. Nutrition, *59:* 225.

Dietary Fat and Protein and Serum Cholesterol

I. ADULT SWINE[1]

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Although atherosclerosis can be produced in a variety of animal species, the specific morphologic counterpart of human atherosclerosis has not been developed (Food and Nutrition Board Committee on Fats in Human Nutrition, '58). Largely as a result of the report of Gottlieb and Lalich ('54) that approximately one-third of swine reaching the age of three years had sclerotic aortas, more detailed biochemical and pathologic studies with this species have been of interest. In order to provide a basis for the establishment of investigations dealing with lipid metabolism and the atherosclerotic process, a cursory examination of certain dietary influences upon serum cholesterol in swine has been undertaken.

EXPERIMENTAL

Adult female swine, three to 7 years of age, that were obtained as discarded breeder sows were used in this study. All pigs were purebred Yorkshires and weighed 300 to 500 pounds. They were caged individually indoors, but without any attempt at close temperature control. Feed was provided in two equal portions each day to supply a constant daily caloric intake throughout all of the studies and so as to maintain body weight. Water was provided only at feeding time. Blood samples were taken at weekly intervals by lightly clipping off the end of the tail. The blood was permitted to clot and the resulting serum which frequently was slightly hemolyzed was used for cholesterol analysis. Sera for electrophoretic analysis of plasma proteins was taken by needle puncture of the superior vena cava.

In the rat experiments, 25 male albino rats[2] of an average weight of 280 gm were caged individually in raised-wire screen-bottom cages and were given food and water ad libitum. They were weighed weekly and blood samples were taken at three-week intervals by heart puncture under light ether anesthesia. During the first 6-week period (low fat) the average weight increased to 410 gm. For the remainder of the study (18 weeks) average weight increased to 480 gm.

The diet employed in the first experiment was a reasonable duplication of the food ingredients used in a human experiment that is being reported separately. The diet composition given in table 1 incorporated, insofar as was practical, the same food ingredients in the same amounts that were utilized in the corresponding study in man. The various components were passed through a meat grinder and the slurry was thoroughly mixed in a large Hobart mixer. The one dietary ingredient that was most subject to variability was the lean ground beef. This was purchased as a single lot and after mixing was packaged in individual weekly portions and frozen. The rat food was made up in the same manner as the pig diet, but without added cerelose or fat and was then lyophilized. The dried mixture was ground and the various diets prepared by adding either sucrose or fat. Cellulose[3] was added to the fat diets so as to maintain a constant caloric density. In this respect the pig and rat diets differed, but the pigs were

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[2] Holtzman, Madison, Wisconsin.

[3] Solka Floc.

TABLE 1

Diet composition

Human-type, mixed diet			
	gm		*gm*
Skim milk powder	3,180	Cabbage	2,14[illegible]
Dried whole egg	387	Carrots	85[illegible]
Wheat flour (all purpose)	4,132	Fish (frozen ocean perch)	8,59[illegible]
Sugar (sucrose)	1,817	Bread (Italian)	1,07[illegible]
Corn flakes	1,289	Beef (ground)	6,36[illegible]
Grapefruit juice (canned)	5,157	Potatoes (cooked)	4,29[illegible]
Vinegar	184	String beans	3,43[illegible]
Spinach (canned)	3,009	Cerelose (low-fat diet only)	
Green pepper	430	Fat or oil	3,63[illegible]
		Total	50,00[illegible]

Purified diet (low fat)

Major component	High protein	Low protein	Protein-free
Soybean protein[1]	15	6	—
Glucose (cerelose)	77[2]	86[2]	92[2]
Cellulose[3]	2	2	2
Minerals[4]	4	4	4
Fat-soluble vitamins in corn oil[5]	2	2	2
B vitamins in glucose[6]	0.15	0.15	0.1[illegible]
Choline dehydrogen citrate	0 22	0.22	0.22
Totals	100.37	100.37	100.3[illegible]

[1] Isolated soybean protein, Promine, supplied by Central Soya Inc., Chicago.
[2] In fat-containing diets 17.1 parts of fat replaced an equivalent caloric quantity of glucose.
[3] Solka Floc.
[4] Mineral mixture per 100 lbs. of diet: $CaH\,PO_4 \cdot 2H_2O$, 350.8 gm; $CaCO_3$, 474.8 gm; KH_2PO_4, 522 gm; NaCl, 227.3 gm; $CuSO_4 \cdot 5H_2O$, 786 mg; $FeSO_4 \cdot 7H_2O$, 7470 mg; KI, 13 mg; $MnSO_4 \cdot H_2O$, 5526 mg; $CoCl_2 \cdot 6H_2O$, 364 mg; $MgCO_3$, 174 gm; $ZnSO_4 \cdot 7H_2O$, 22 gm.
[5] Fat-soluble vitamins per 100 lbs. of diet: vitamin A acetate, 6,500,000 I.U.; Calciferol, 6.75 mg; α-tocopherol, 9,000 mg; corn oil, 1.0 kg.
[6] B vitamins per 100 lbs. of diet: thiamine·HCl, 1899 mg; riboflavin, 3,600 mg; niacin, 15,000 mg; Ca pantothenate, 14,669 mg; pyridoxine·HCl, 2,185; cobalamine, 15 mg; folic acid, 900 mg; menadione, 1,500 mg; cerelose, 2.0 kg.

fed a constant caloric amount of food while the rats ate ad libitum. Butter was added to the diets with a correction applied for the non-fat portion.

The fat content of the diets is shown in table 2. The energy value of the wet pig diet was calculated to be 237 Cal. per 100 gm and the lyophilized rat diet with either sucrose or fat, 437 Cal. per 100 gm. In both diets fat supplied approximately 27% of the calories.

In the second swine experiment purified diets containing various amounts of protein[4] were used. The composition of these diets is given in table 1.

Cholesterol was determined by the method of Abell et al. ('52).

[4] Promine, isolated soybean protein, Central Soya Inc., Chicago, Illinois.

TABLE 2

Fat and protein content of diets

Diet	Fat analysis
	%
Human-type mixed diet (Analysis on wet basis, as fed to pigs)	
Low fat	1.2
Olive oil	6.9
Corn oil	7.1
Butterfat	7.1
Hydrogenated fat	6.7

Diet	Fat	Protein N × 6.25
	%	%
Purified diet—low fat		
High protein	1.9	13.7
Low protein	2.1	4.9

RESULTS

Mixed diets of natural foods—swine and rats

Figure 1 (upper) illustrates the changes in blood serum cholesterol of swine and rats when alterations in the type of fat were introduced into the basal diet that was patterned after a human food mixture. The swine showed some erratic changes, but in general the rise and fall

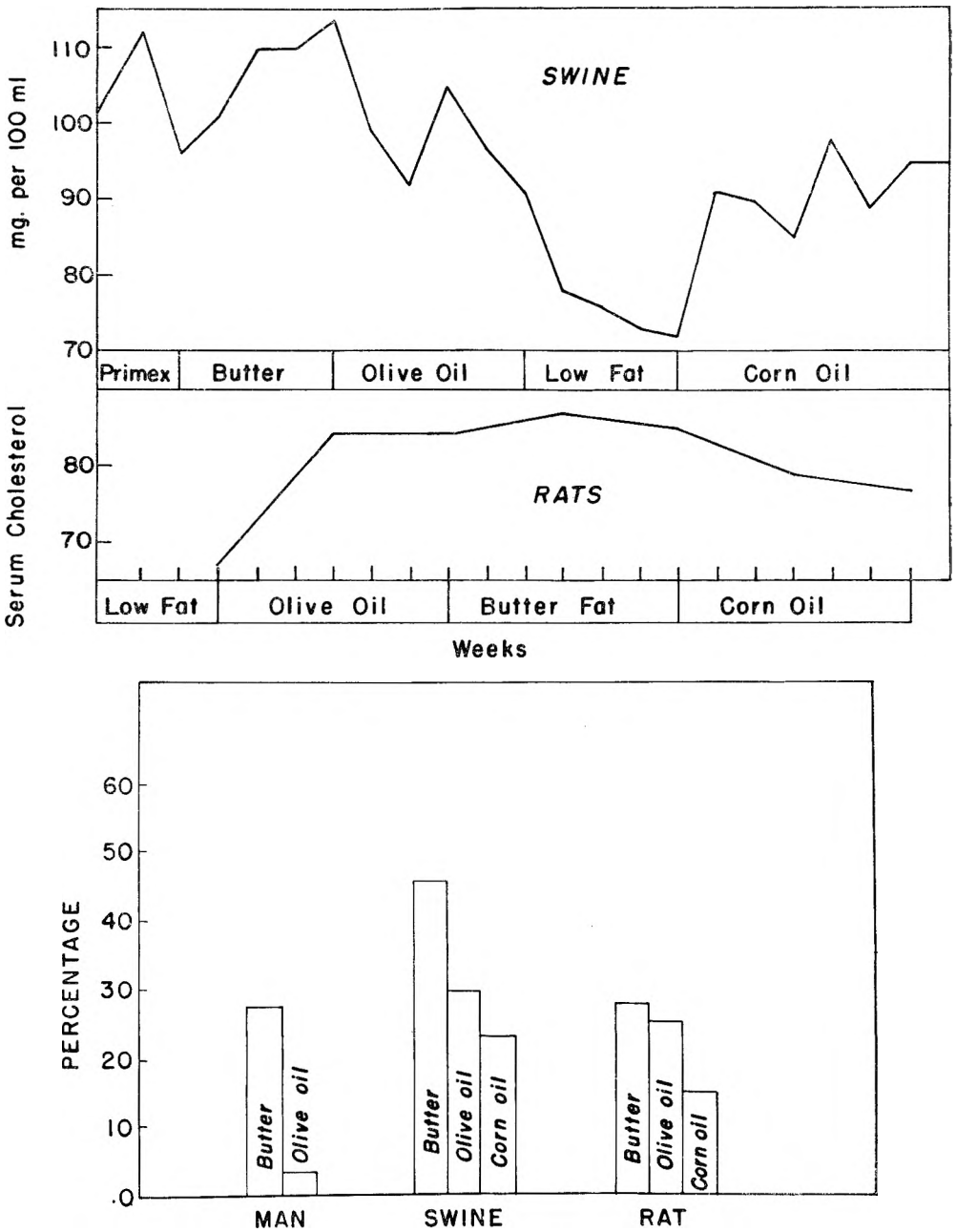

Fig. 1 Top, serum cholesterol changes in swine and rats receiving a diet of mixed-natural foods in which different fats were incorporated. Bottom, percentage changes of serum cholesterol from low fat diets.

of serum cholesterol followed the pattern that would be expected from the many reports of studies in man. Hydrogenated fat, and particularly butterfat, resulted in higher cholesterol values than the unsaturated vegetable fats or the low-fat diet. Rats exhibited smaller changes in serum cholesterol due to the dietary fats, but still differences between fats of varying degrees of unsaturation were observed and the lowest values were obtained with the low-fat diet. Only one serum cholesterol for the low-fat diet was taken and this represented the average of the 25 rats at the end of three weeks on this regimen. If average values for each fat period are expressed as a percentage change from the low-fat regimen the effects of the different fats in man, swine and rats as shown in figure 1 (lower) were obtained. The results in man are taken from data that are being published separately. While corn oil was not studied in human subjects in the present series, there are several reports in the literature that show this fat gives as low or perhaps even lower cholesterol values than are obtained with low-fat diets (Beveridge et al., '56; Malmros and Wigand, '57). The effect of corn oil in man is further illustrated in the study of Ahrens et al. ('57) where it was shown that increasing this fat in the diet from 15 to 70% had little if any influence upon the level of serum cholesterol. Obviously this was not the case with swine or rats and from a perusal of the literature apparently this condition is not obtained with any experimental animal species. For example, rats (Avigan and Steinberg, '58) cockerels (Stamler et al., '57) monkeys (Portman et al., '56) and rabbits (Diller et al., '58) show an increase in serum cholesterol when corn oil is added to a low-fat diet or in some cases when the amount of corn oil in the diet is increased. Aside from this one outstanding difference both swine and rats showed the same qualitative response to unsaturation that is found in man, but swine exhibited a greater differentiation in their reaction to fats of varying degrees of unsaturation than was seen in rats.

Purified diets—effect of protein and fat

Two groups of 5 adult sows each were fed the purified diet described in table 1. One group received the high-protein diet (13.7%) for the duration of the study with changes in the dietary fat being made as shown in figure 2 (upper). The second group received the low-protein diet (4.9%) for the first part of the study, but two protein-free periods were introduced toward the end of the study. The sequence of diet changes is shown in this chart. In order to compare the serum cholesterol responses to the different fats at the three levels of protein intake the average cholesterol values for each diet period are shown in figure 2 (lower). The low fat and three different types of fat gave characteristic serum cholesterol responses. Serum cholesterol values were not influenced by protein levels in the diet of 13.7 or 4.9%. There was no effect observed during the first 4-week period when all of the protein was removed and corn oil was continued as the dietary fat. However, during a second 4-week period when the fat was changed to beef tallow a rise in serum cholesterol above the level obtained with the high-protein diet was observed. This cholesterol elevation continued even when protein at the 4.9% level was returned to the diet.

Prior to removing all of the protein from the diet, serum protein and albumin levels (paper electrophoresis) were normal. Average values for the 13.7% protein group were: total serum protein, 6.3%; albumin, 32% of the total. In the 4.9% protein group the values were: total serum protein, 6.0%; albumin, 29% of the total. These values are lower than those reported for normal swine by Knill et al. ('58). Satisfactory blood samples for electrophoretic analysis were not obtained during the protein-free periods so that one can only surmise the rate of onset and the degree of severity of protein depletion that took place.

Finally, a comparison of serum cholesterol responses in swine receiving the mixed-food diet and the high-protein purified diet is given in figure 3. In each instance the natural mixed-food diet gave

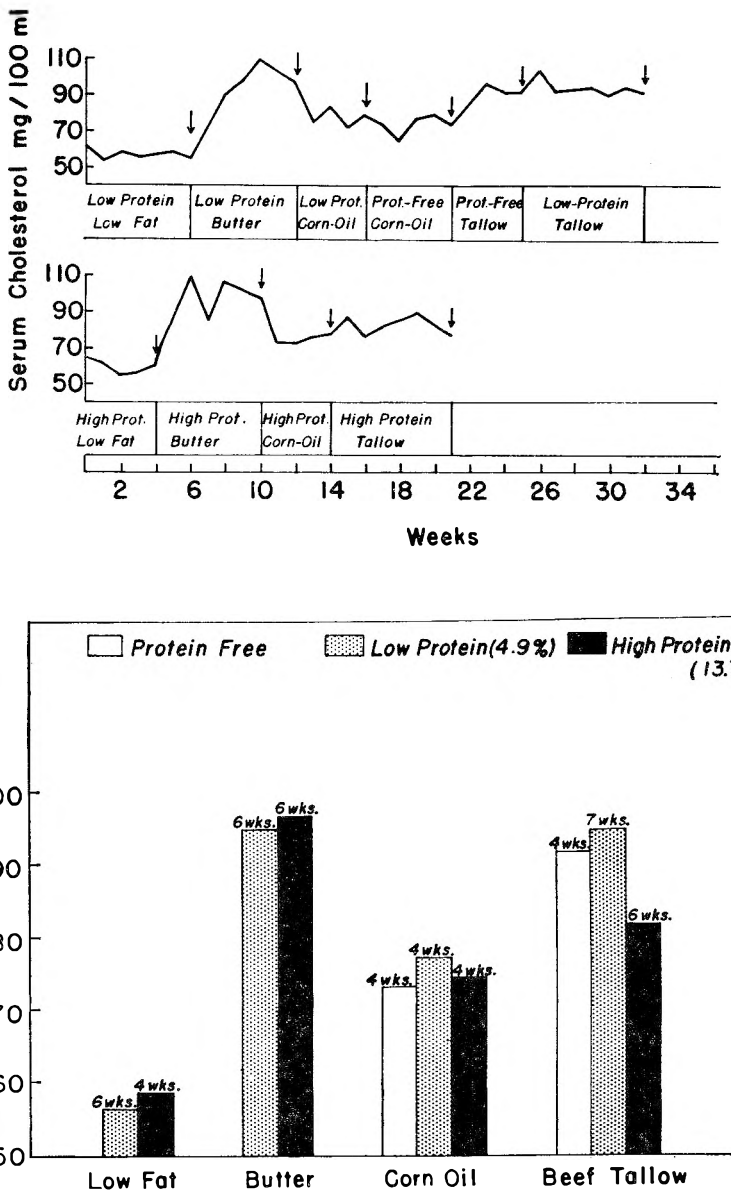

Fig. 2 Top, effect of changes in dietary fat and protein upon serum cholesterol of adult swine fed purified diets. Bottom, serum cholesterol averages for periods as shown at top.

higher blood cholesterols, but the differences between the two types of diet appear to be approximately constant. In other words, the serum cholesterol response to the amount and type of fat fed is not affected qualitatively by the kind of food mixture being ingested, but quantitatively the cholesterol values are higher when normal human-type foods are fed. It should be kept in mind that several of the natural foods contributed significant quantities of cholesterol. In fact an analysis of the low-fat mixture (wet weight) showed 0.019% of cholesterol.

DISCUSSION

Apparently many animal species with the exception of man increase blood cholesterol level when fat, regardless of the degree of unsaturation, is fed. This is not the only lack of parallelism that is found between man and other species that is closely related to the problem of atherosclerosis and heart disease. However, it is particularly distressing in searching for clues that will help in understanding the relationship between nutrient intake and these disease processes. Accepting this obvious shortcoming, the work with swine that is reported here does give encouragement that this species may be of help in unraveling some of the important interrelationships. Aside from such advantages as availability of inbred strains, ease of handling and adaptability to extremes of dietary manipulations, swine do show characteristic blood cholesterol responses that correspond in some degree to observations made in man.

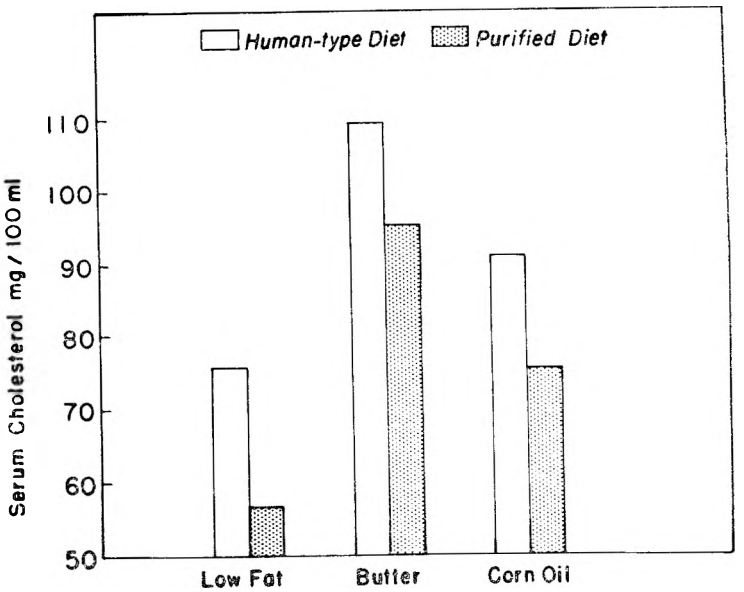

Fig. 3 Comparison of the serum cholesterol response to dietary fat in adult swine receiving either the mixed-natural food diet or a purified diet.

There was no apparent difference in the cholesterol responses to different fat intakes in the swine when the fat was incorporated in a diet of natural foodstuffs or in a highly purified diet. The consistently higher serum cholesterols obtained with the natural food diet may be accounted for by the cholesterol content of that diet.

Three laboratories have reported preliminary results of the effect of dietary fat upon serum cholesterol in swine. In one of these (Bragdon et al., '57) no significant effect of added corn oil in the diet was observed although the average values were elevated, but butter caused a definite increase. In a second study (Pfeifer and Lundberg, '58) both corn oil and beef tallow raised the serum cholesterol and with maintenance intake of total calories the beef tallow gave slightly higher values than corn oil. In the third study (Roswell et al., '58), swine were fed either a low-fat or isocaloric diets rich in butter or margarine. Blood cholesterol did not increase with either the margarine or butter diets.

Results that have been reported on the influence of dietary protein are divergent. For example, Keys and Anderson ('57) varied the protein intake of a group of human subjects from 65 to 132 gm per day without detecting any significant effect upon serum cholesterol. On the other hand, in 9 human subjects Olson et al. ('58b) reported a hypocholesteremic response due to the lowering of dietary protein intake from 100 to 25 gm per day for one week. At the 25 gm level all of the dietary protein was of vegetable origin. From one nitrogen balance study they conclude: "The degree of hypocholesterolemia did not parallel the degree of negative nitrogen balance and hence it seems unlikely that the effect upon serum lipids is due to a net catabolism of body protein." In the rat, Jones and Huffman ('56) found that young adults (three months post weaning) showed a rise in serum cholesterol when the dietary protein was either raised or lowered beyond an intermediate

point of 12 to 18%. The regimens had to be continued for several months in order to obtain stabilized results. Nath et al. ('58) have confirmed this diphasic response to protein, but they used young rats and developed an artificial hypercholesterolemia by feeding cholesterol and cholic acid. With casein their cholesterol values were lower at 40% than at 60 or 70%. This type of response was not seen when wheat gluten was used as the source of protein. Contrary to these observations Olson et al. ('58a) have reported a hypocholesterolemic response to low-protein, low-choline diets in young adult rats. Moyer et al. ('56) had previously found that with cholesterol and cholic acid in the diet rats showed progressively decreasing serum cholesterol values as the protein in the diet was increased. In chicks at 4 weeks of age Johnson et al. ('58) found decreasing cholesterol values as the diet protein was increased. This trend held for chicks receiving diets either with or without added cholesterol. Nikkila and Ollila ('58) observed this same inverse relationship between dietary protein and serum cholesterol when 1.5% of cholesterol was present in the diet, but not in its absence. Leveille and Fisher ('58) and Kokatnur et al. ('58) have both reported a hypercholesterolemic effect of low-protein diets. In the latter of these two studies mature chickens were used and no agents such as cholic acid or cholesterol were fed. In this instance minimal serum cholesterol changes were observed and the largest increase was noted in those birds that consumed the least amount of protein. With the exception of the studies of Olson and coworkers, all of the above investigators have found that if the protein intake was low enough, a hypercholesterolemic response was obtained. The amount of protein appears to be more critical in the growing animal than in the adult. The work that is being reported with swine tends to confirm this relationship. A hypercholesterolemic effect was observed only when the protein content of the diet was reduced below 5%. However, from electrophoretic analysis of the serum proteins there was no evidence of protein deficiency when 5% protein diets were consumed, and apparently more than 4 weeks on a protein-free diet was required to induce a severe protein depletion. Hypercholesterolemia of young, rapidly growing swine has been observed with relatively small changes in protein intake and is being reported separately (Barnes et al., '59). It would appear that protein at decreasing levels in the diet becomes critical in the establishment of a hypercholesterolemia only when evidence of protein deficiency is manifested. This, of course, will be dependent upon many factors such as animal species, duration of study, age, protein composition, composition of diet and many others.

The liproprotein pattern in swine as determined by paper electrophoresis is of some interest. Boyd and Oliver ('56) state that the ratios of α to β lipoproteins in several species are as follows: rat, 70:30; rabbit, 50:50; man (normal young males), 28:72. Bragdon et al. ('57) reported that by ultracentrifugal analysis normal swine sera has a concentration of low density lipoprotein of 85 mg per 100 ml and of high density lipoproteins 207 mg per 100 ml. In man this ratio is reversed, but in all other animal species studied the ratios were in the same direction as found in swine. In the authors' laboratory swine receiving a corn oil diet have shown an average ratio of α to β lipoproteins by paper electrophoresis of 37:63. This, of course, is in the same direction as has been reported for man and apparently differs from most other species. As a check, simultaneous ratios were determined for a group of 12 healthy young men and was found to be 25:75. This indicates another important biochemical similarity between man and swine and should be studied further.

SUMMARY

Adult swine were fed either a human-type diet of mixed foods or a purified diet with changes in the amount and type of fat or the amount of protein. In both types of diet, fat caused the serum cholesterol to rise with the greatest increase resulting from the most saturated fat. The human-type diet consistently gave higher serum cholesterol values, but the response to different fats paralleled the

results obtained with the purified diet. With the purified diet, lowering the protein to a level of approximately 5% did not affect the level of serum cholesterol. However, with the large sows (300 to 500 pounds) that were used, this protein level gave no indication of depletion of serum proteins. When all protein was removed from the diet and the swine were continued on this regimen for more than 4 weeks some elevation of serum cholesterol was observed. Swine receiving a corn oil diet had lipoprotein values by electrophoretic analysis in which the α fraction was consistently lower than the β. This is the same direction as ratios that have been found in man.

LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. H. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

Ahrens, E. H., Jr., J. Hirsch, W. Insull, Jr., T. T. Tsaltas, R. Blomstrand and M. L. Peterson 1957 The influence of dietary fats on serum-lipid levels in man. Lancet, *272:* 943.

Avigan, J., and D. Steinberg 1958 Effects of saturated and unsaturated fat on cholesterol metabolism in the rat. Proc. Soc. Exp. Biol. Med., *97:* 814.

Barnes, R. H., E. Kwong, W. Pond, R. Lowry and J. K. Loosli 1959 Dietary fat and protein and serum cholesterol. II. Young swine. J. Nutrition, *69:* 269.

Beveridge, J. M. R., W. F. Connell and G. A. Mayer 1956 Dietary factors affecting the level of plasma cholesterol in humans: The role of fat. Can. J. Biochem. Physiol., *34:* 441.

Boyd, G. S., and M. F. Oliver 1956 Some aspects of cholesterol metabolism. Proc. Nutrition Soc., *15:* 52.

Bragdon, J. H., J. H. Zeller and J. W. Stevenson 1957 Swine and experimental atherosclerosis. Proc. Soc. Exp. Biol. Med., *95:* 282.

Diller, E. R., B. L. Woods and O. A. Harvey 1958 Endogenous hypercholesterosis in rabbits fed a purified diet and the effect of unsaturated lipid. Federation Proc., *17:* 212.

Food and Nutrition Board Committee on Fats in Human Nutrition 1958 The role of dietary fat in human health. National Academy of Sciences—National Research Council, Washington, D. C. Publication 575.

Gottlieb, H., and J. J. Lalich 1954 The occurrence of arteriosclerosis in the aorta of swine. Am. J. Path., *30:* 851.

Johnson, D., Jr., G. A. Leveille and H. Fisher 1958 Influence of amino acid deficiencies and protein level on the plasma cholesterol of the chick. J. Nutrition, *66:* 367.

Jones, R. J., and S. Huffman 1956 Chronic effect of dietary protein on hypercholesteremia in the rat. Proc. Soc. Exp. Biol. Med., *93:* 519.

Keys, A., and J. T. Anderson 1957 Dietary protein and the serum cholesterol level in man. Am. J. Clin. Nutrition, *5:* 29.

Knill, L. M., T. R. Podleski and W. A. Childs 1958 Normal values of swine serum proteins. Proc. Soc. Exp. Biol. Med., *97:* 224.

Kokatnur, M., N. T. Rand, F. A. Kummerow and H. M. Scott 1958 Effect of dietary protein and fat on changes of serum cholesterol in mature birds. J. Nutrition, *64:* 177.

Leveille, G. A., and H. Fisher 1958 Plasma cholesterol in growing chicken as influenced by dietary protein and fat. Proc. Soc. Exp. Biol. Med., *98:* 630.

Malmros, H., and G. Wigand 1957 The effect on serum-cholesterol of diets containing different fats. Lancet, *273:* 1.

Moyer, A. W., D. Kritchevsky, J. B. Logan and H. R. Cox 1956 Dietary protein and serum cholesterol in rats. Proc. Soc. Exp. Biol. Med., *92:* 736.

Nath, N., A. E. Harper and C. A. Elvehjem 1958 Dietary protein and serum cholesterol. Arch. Biochem. Biophys., *77:* 234.

Nikkila, E. A., and O. Ollila 1958 Effect of low protein diet on the serum lipids and atherosclerosis of cholesterol-fed chickens. Am. J. Clin. Nutrition, *6:* 95.

Olson, R. E., J. R. Jablonski and E. Taylor 1958a The effect of dietary protein fat and choline upon the serum lipids and lipoproteins of the rat. Am. J. Clin. Nutrition, *6:* 111.

Olson, R. E., J. W. Vester, D. Gursey, N. Davis and D. Longman 1958b The effect of low-protein diets upon serum cholesterol in man. Ibid., *6:* 310.

Peifer, J. J., and W. O. Lundberg 1958 Influence of total calories, fat calories and fat unsaturation on blood lipides. Federation Proc., *17:* 288.

Portman, O. W., D. M. Hegsted, F. J. Stare, D. Bruno, R. Murphy and L. Sinisterra 1956 Effect of the level and type of dietary fat on the metabolism of cholesterol and beta lipoproteins in the cebus monkey. J. Exp. Med., *104:* 817.

Roswell, H. C., H. G. Downie and J. F. Mustard 1958 The experimental production of atherosclerosis in swine following the feeding of butter and margarine. Canad. Med. Assoc. J., *79:* 647.

Stamler, J., R. Pick and L. N. Katz 1957 Effects of various fats on cholesterolemia and atherogenesis in cholesterol-fed cockerels. Federation Proc., *16:* 123.

Dietary Fat and Protein and Serum Cholesterol

II. YOUNG SWINE[1]

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Recently reported results from the authors' laboratories have indicated certain interrelationships between dietary fat and protein and serum cholesterol levels in adult swine (Barnes et al., '59). It was found that extreme conditions of protein deprivation were necessary in order to demonstrate an elevation of the serum cholesterol. Since the rapidly growing animal is very sensitive to relatively small changes in dietary protein level it was of interest to extend these studies to the young pig.

EXPERIMENTAL

Sixteen boars from 4 litters were weaned at 8 weeks of age and divided into 4 groups so as to distribute litter mates equally in all groups. They were placed in individual concrete-floor pens and fed ad libitum until their weight reached 200 pounds. Upon reaching this weight, each pig was transferred singly to controlled feed intake at 3.5 pounds per day until attaining a weight of 250 pounds. From then on, 5 pounds of feed per day was provided. Growth curves in figure 1 show values up to the time that a boar in any group reached 200 pounds. Since feeding conditions in a group changed at that point comparative growth rates would have an altered significance. The 4 diets are described in table 1. The diets were designed so that by manipulation of corn, soybean oil meal and meat scraps either a high-protein (approximately 16% by analysis) or low-protein (approximately 9% by analysis) content was achieved. Then by the addition of 10% of beef tallow to one high-protein and one low-protein diet, high-fat (approximately 13% by analysis) or low-fat (approximately 3% by analysis) diets were made. Very small quantities of cholesterol may have been provided by the meat scraps and beef tallow, but obviously such contamination would be of a different order of magnitude from the quantities of cholesterol that are normally fed so as to induce a hypercholesterolemia. Blood samples were taken at 4-week intervals either from the superior vena cava or by clipping the tail. Serum cholesterol was determined by the method of Abell et al. ('52). Serum proteins were determined in a few of the pigs at 32 weeks by the biuret reaction as developed by Gornall et al. ('49) and by paper electrophoresis. Lipoprotein patterns were measured by the electrophoretic procedure of Jencks et al. ('55, '56).

RESULTS

Growth curves during the ad libitum feeding period are shown in figure 1. The two low-protein groups had retarded growth and lowered feed efficiency as would be expected. However, the high-fat, low-protein group was particularly depressed. At 20 weeks these boars exhibited not only stunted growth, but were generally unthrifty in appearance with rough hair coat and had developed stiff or sore joints so that they had difficulty in standing or walking. After a total of 32 weeks these signs had largely disappeared, but, of course, they remained stunted in growth. At the end of the experiment (41 weeks) all pigs were on controlled feed intake

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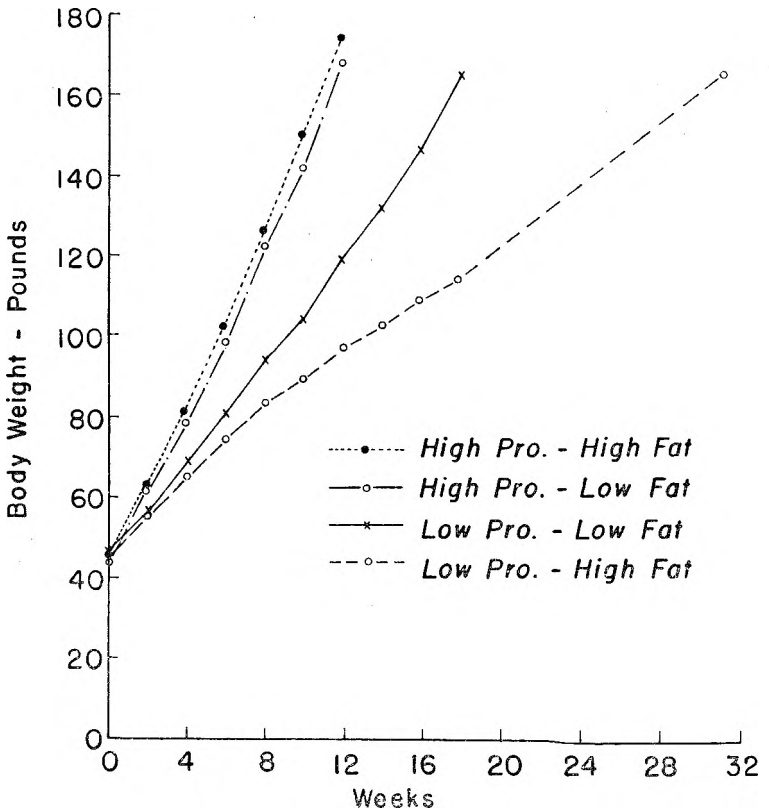

Fig. 1 Growth of young swine during periods of ad libitum feeding. The growth curves have been terminated when one or more animals in a group reached a weight of 200 pounds.

TABLE 1

Diet composition

	Low protein–low fat	Low protein–high fat	High protein–low fat	High protein–high fat
Corn	87.45	77.45	69.45	59.45
Soybean meal	2.50	2.50	19.00	19.00
Meat scraps	2.50	2.50	5.00	5.00
Alfalfa meal	5.00	5.00	5.00	5.00
Dicalcium phosphate	1.50	1.50	1.50	1.50
Salt[1]	0.50	0.50	0.50	0.50
Vitamins[2]	0.05	0.05	0.05	0.05
Aureomycin[3]	0.50	0.50	0.50	0.50
Stabilized beef tallow	—	10.00	—	10.00
Total[4]	100.00	100.00	100.00	100.00

[1] Sodium chloride with trace amounts of iodine, cobalt, iron and copper added.

[2] Fortafeed 2–49 C, American Cyanamid Co., New York, N. Y., supplies 1, 2, 4.5, 5 and 0.03 mg of riboflavin, pantothenic acid, niacin, choline chloride and folic acid per pound of feed, respectively.

[3] Aurofac 2A, American Cyanamid Co., New York, N. Y., supplies 18 mg aureomycin per pound of feed.

[4] Zinc carbonate added to each ration to supply zinc at the rate of 54 ppm and vitamins A and D added to supply 400 and 90 I.U. per pound of feed, respectively.

except two in the low-protein, high-fat group. The equalization of feed intake had a tendency to equalize body weight in the three other groups. The final body weight averages were: low-protein, low-fat, 281; low-protein, high-fat, 209; high-protein, low-fat, 272; high-protein, high-fat, 309.

Values for total serum cholesterol are shown in figure 2. The first determination was run after the pigs had been on the experimental diets for about a week and the effect of the dietary fat in raising the serum cholesterol was already established. A marked effect of protein was manifested by the end of the first month. All groups showed a rise in serum cholesterol reaching maximal values after 4 to 8 weeks followed by a decline which appeared to be

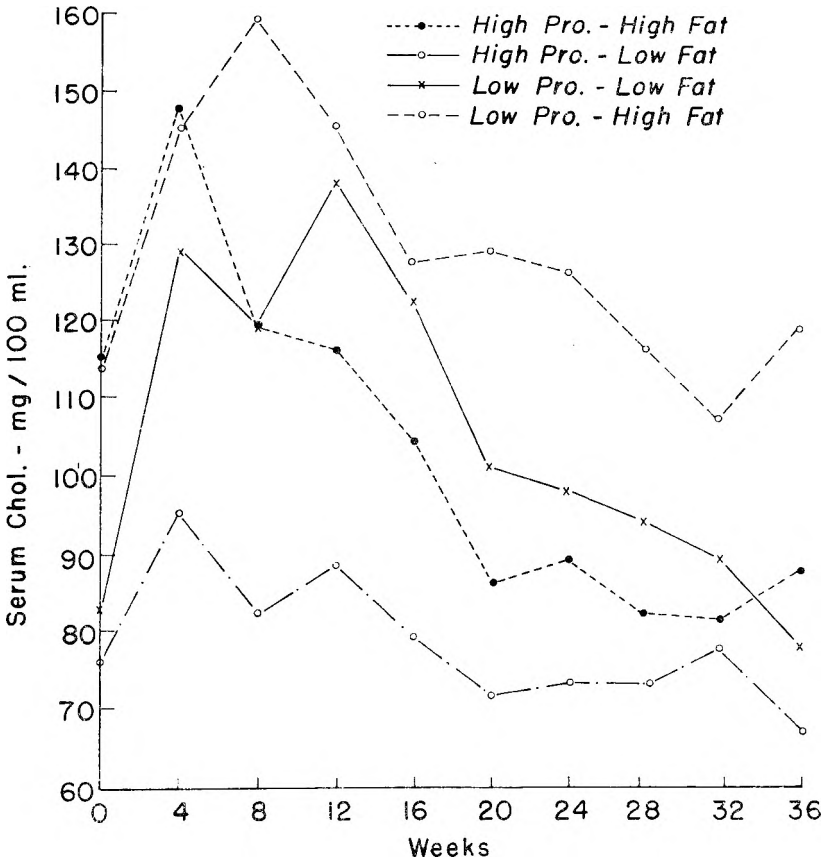

Fig. 2 Serum cholesterol changes of young swine for 36 weeks post-weaning. The animals had been on the experimental diets for approximately one week prior to zero time shown on these curves.

TABLE 2

Serum proteins at 36 weeks

Type of diet	Serum proteins			Serum lipoproteins		
	No. analyses	Total	Albumin	No. analyses	α	β
		gm/100 ml	*% of total*		%	%
Low protein, low fat	1	6.8	36.7	2	33	67
Low protein, high fat	4	6.7	23.6	2	35	65
High protein, low fat	1	—	40.0	2	33	67
High protein, high fat	1	8.3	39.8	1	47	53

approaching minimal levels. The two high-protein groups reached minimal constant levels of serum cholesterol most quickly while the low-protein groups lagged behind. It is obvious that both high-fat– and low-protein-containing diets were responsible for increasing the serum cholesterol levels and the magnitude of effect for both nutrients was greatest during the most active growth period.

The retarded growth rates of the two low-protein groups is *a priori* evidence of a relative deficiency of protein. The more marked deficiency in the high-fat group is further demonstrated by the serum protein values taken at the 32 weeks that are shown in table 2. Both low-protein groups appear to have depressed total protein and albumin, but the serum albumin level in the low-protein, high-fat group was most strikingly decreased. The ratio of α to β lipoproteins was constant except for the high-protein, high-fat groups which showed an increase in the α fraction. This may have been an anomaly since only one pig was bled for this determination. The fact that the ratios are similar to those found in normal human serum is of considerable interest. An average of 12 normal males gave an average ratio of α to β lipoproteins of 25:75.

DISCUSSION

The severity of protein deprivation in the pigs receiving the low-protein, high-fat diet is of particular interest. It seems logical that this condition was due in part to a decrease in the percentage of protein calories in the diet caused by increasing the caloric density of the diet with added fat. Several preliminary reports from the Human Nutrition Research Unit, National Institute for Medical Research in London describe the development of a kwashiorkor-like syndrome in young pigs fed low-protein diets (Knowles, '57; Stewart and Platt, '58; Heard et al., '58). With knowledge of the British findings it seems quite probable that the present studies provide another example of a kwashiorkor-like syndrome in the young pig. In this instance a high calorie to protein ratio was provided by additional fat in the diet. In the British studies this ratio was maintained high by feeding extra carbohydrate. Kwashiorkor is not readily duplicated in experimental animals and perhaps this is due in part to the fact that animals fed a low-protein diet ad libitum have a decreased food intake and, therefore, do not maintain a sufficiently high calorie intake associated with low protein. Perhaps due to the voracious appetite of the pig coupled with a fairly high caloric density diet the conditions for this severe form of protein malnutrition were achieved. The protein level in the diet was not thought to be critically low (approximately 9%) and probably was sufficient to permit some remission of symptoms. This species may prove to be of considerable value in the study of severe protein malnutrition.

The pattern of serum cholesterol changes was most unusual and unexpected. The precipitous rise during the early months of growth was greatly exaggerated by the fat in the diet and also the presence of protein deficiency. These findings demonstrate that very high serum cholesterol values with low-fat diets can occur, but only during the time that protein is seriously limiting in the diet. The results at the 36th week seem to be approaching the condition that has been described in adult swine (Barnes et al., '59) where protein even at a level of 5% in the diet was found to be without effect upon serum cholesterol. The exception is in the case of the low-protein, high-fat group where, although declining gradually, the level remains far above that of the other three groups.

Some of the key literature dealing with the relationship between dietary protein and serum cholesterol has been reviewed in the preceding paper (Barnes et al., '59). It has been noted that growing chickens show extreme elevation of cholesterol when a low-protein diet was fed (Leveille and Fisher, '58). However, this was under conditions of cholesterol feeding where an artificial hypercholesterolemia was produced. The present study demonstrates the same effect although transient in the absence of hypercholesterolemic agents and in addition illustrates the extreme importance of the time relationship.

The ratio of α to β lipoprotein seems to be unrelated to the level of serum cholesterol. In man it is generally observed that

hypercholesterolemia is associated with an increase in the β fraction or perhaps more specifically an increase in the cholesterol of this fraction (Kritchevsky, '58). The high-protein, high-fat group appears to show the reverse change, but only one observation was made and its significance may be in doubt.

SUMMARY

Four groups of weanling pigs were fed either high- or low-protein and either high- or low-fat diets for 36 weeks. Evidence of protein malnutrition was most marked in the low-protein, high-fat group. These animals exhibited signs that have been interpreted as resembling the human infant disease, kwashiorkor.

Fat in the form of beef tallow in the diet caused a rapid rise in serum cholesterol. Low-protein intakes also resulted in increase in serum cholesterol. The cholesterol values reached a peak between the 4th and 8th week and then declined slowly toward the levels found in adult swine. The low-protein, high-fat group did not return toward the minimal level as rapidly as the other groups, and this has been related to the greater severity of protein malnutrition that they exhibited.

LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

Barnes, R. H., E. Kwong, G. Fiala, M. Rechcigl, R. N. Lutz and J. K. Loosli 1959 Dietary fat and protein and serum cholesterol. I. Adult swine. J. Nutrition, *69:* 261.

Gornall, A. G., C. J. Bardawill and M. M. David 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., *177:* 751.

Heard, C. R. C., B. S. Platt and R. J. C. Stewart 1958 The effects on pigs of a low-protein diet with and without additional carbohydrate. Proc. Nutrition Soc., *17:* xli.

Jencks, W. P., E. L. Durrum and M. R. Jetton 1955 Paper electrophoresis as a quantitative method: The staining of serum lipoproteins. J. Clin. Invest., *34:* 1437.

Jencks, W. P., M. R. Hyatt, M. R. Jetton, T. W. Mattingly and E. L. Durrum 1956 A study of serum lipoproteins in normal and atherosclerotic patients by paper electrophoretic techniques. Ibid., *35:* 980.

Knowles, C. B. 1957 Protein malnutrition in the pig. Proc. Nutrition Soc., *16:* ix.

Kritchevsky, D. 1958 Cholesterol. John Wiley & Sons, Inc., New York.

Leveille, G. A., and H. Fisher 1958 Plasma cholesterol in growing chicken as influenced by dietary protein and fat. Proc. Soc. Exp. Biol. Med., *98:* 630.

Stewart, R. J. C., and B. S. Platt 1958 Arrested growth lines in the bones of pigs on low-protein diets. Proc. Nutrition Soc., *17:* v.

Relation between Dietary Fat, Fat Content of Milk and Concentration of Certain Enzymes in Human Milk

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Studies were recently undertaken in this laboratory to determine whether differences in socioeconomic status are associated with differences in nutritional status, and if so, whether such differences affect the quality of human milk. It was found that increases in dietary fat up to a certain amount are accompanied by increases in the fat content of breast milk (Karmarkar, Deodhar, Kapur and Ramakrishnan, '58; Karmarkar, Kapur, Deodhar and Ramakrishnan, '59). It has been suggested that human milk contains certain enzymes like lipase (Jacqmain and Loncin, '53; Arshavskii, '40) and phosphatase (Chanda and Owen, '51; Vittu, '46) which may influence the health and nutrition of newborn infants and that the phosphatase concentration in milk is possibly related to its fat content (Stewart, Platou and Kelly, '58). An attempt was therefore made to determine the correlation between dietary fat and milk fat and to investigate whether the fat content of milk affects the concentration of enzymes like lipase, esterase, alkaline and acid phosphatase in milk. The results of this investigation are reported in this paper.

EXPERIMENTAL

Materials and methods. The subjects of this investigation were 60 lactating women of normal health living in and around Baroda. Since it has been found by Stewart, Platou and Kelly ('58) that the lactation period affects the alkaline phosphatase content of human milk, the subjects were chosen from the same lactation period, namely, three to 4 months.

Collection of diet and analysis of its fat content. The whole day's diet of the subjects was obtained by making collections of equal amounts of all the foodstuffs consumed by the subject including snacks, beverages, and dietary supplements, if any, in the form of vitamins, minerals, etc.

For analysis of food, a separate weighing was made of each foodstuff and all the foodstuffs were then thoroughly mixed and homogenized. A portion of the homogenized food was dried in an electric oven at 90° for 12 hours. The fat content of the food was estimated according to the method of Banerjee and Biswas ('57). The collection of food taken and the analysis of same for the fat content was carried out for three consecutive days for each subject and the average daily intake of fat was therefrom estimated.

Sampling of milk for analysis. The samples for analysis were collected in all cases between two feedings at about 3 P.M. by voluntary expression. The samples were brought to the laboratory in thermos flasks containing ice powder and analyzed immediately. The sampling was done on three consecutive days and the average value for the three samples was taken.

Estimation of fat in milk. The fat content of milk was estimated according to the method of Chiba, Moriwaki, Suzuki, Hirai and Imahori ('56).

Estimation of lipase in milk. Lipase was estimated by essentially the same method as that of Boissonnas ('48). The assay mixture consisted of 1.5 ml of Tween 20,[1] 2.0 ml of 0.1 M phosphate buffer, pH 6.8, 0.2 ml of milk sample, 0.3 ml of 0.04% phenolphthalein in alcohol, and water to 5.0 ml. Incubation was carried out at 37° for two hours, together with the blank containing boiled enzyme. The reaction was stopped by adding 5.0 ml of absolute alco-

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[1] Tween 20 used for this experiment secured through the courtesy of Atlas Company.

hol after the incubation period. The mixture was warmed in a water bath and titrated against 0.01 N NaOH.

A unit of enzyme activity is defined as the amount of enzyme which, under the described assay conditions, liberates in two hours an amount of free fatty acids that would be neutralized by 1.0 ml of 0.01 N NaOH. Lipase activity is expressed in terms of units per 100 ml of milk sample.

Estimation of esterase in milk. Esterase was estimated by essentially the same method as that of Harrer and King ('41). The assay mixture consisted of 2.0 ml of ethyl butyrate, 1.0 ml of 0.1 M phosphate buffer, pH 7.2, 0.2 ml of milk sample, 0.3 ml of 0.04% phenolphthalein in alcohol, and water to 5.0 ml. Incubation was carried out at 37° for two hours, together with the blank containing boiled enzyme. The reaction was stopped by adding 5.0 ml of absolute alcohol after the incubation period. The mixture was warmed in a water bath and titrated against 0.01 N NaOH.

A unit of enzyme activity is defined in the same way as for lipase. The esterase activity is expressed in terms of units per 100 ml of milk sample.

Estimation of acid and alkaline phosphatases in milk. Acid and alkaline phosphatases were estimated by essentially the same method as that of Morton ('55). The assay mixture consisted of 1.0 ml of 0.1 M sodium β-glycerophosphate, 2.0 ml of 0.1 M acetate buffer, pH 4.4 for acid phosphatase or 2.0 ml of 0.1 M borate buffer, pH 8.6 for alkaline phosphatase, 0.25 ml of 0.2 M magnesium acetate, 0.2 ml of milk and water to 5.0 ml. Incubation was carried out at 37° for 20 minutes and the reaction was stopped by addition of 2.0 ml of 30% trichloracetic acid. The blank containing all components together with 2.0 ml of 30% trichloracetic acid was incubated for the same period. After removing the precipitated protein by centrifugation, the inorganic phosphorus in the supernatant was determined by the method of Fiske and Subbarow ('25).

A unit of enzyme activity is defined as the amount of enzyme which causes the liberation of 1.0 µg of phosphorus in 20 minutes. The acid and alkaline phosphatase activity is expressed in terms of units per 100 ml of milk sample.

RESULTS

The data were classified into a frequency distribution and the median and the quartiles determined for the distribution. The first quartile was 27.5 gm, the median, 50.0 gm, and the third quartile,

TABLE 1

Effect of dietary fat on the fat content as well as lipase, esterase and phosphatase contents of human milk

Classification of subjects	Number of subjects investigated	Mean fat intake per day	Mean fat content of milk	Mean lipase activity	Mean esterase activity	Mean acid phosphatase activity	Mean alkaline phosphatase activity
		gm	*gm/100 ml*	*units/100 ml*	*units/100 ml*	*units/100 ml*	*units/100 ml*
Group 1 (dietary fat, 8.0 to 27.5 gm)	16	18.360	4.125	5461.0	3376.0	1291.1	3279.5
Group 2 (dietary fat, 27.5 to 50.0 gm)	15	37.430	4.300	5762.1	3551.0	922.5	3527.6
Group 3 (dietary fat, 50.0 to 75.0 gm)	16	60.540	4.860	6037.1	3924.8	763.0	3860.8
Group 4 (dietary fat, 72.0 to 115.0 gm)	13	88.980	4.716	6448.7	4305.3	745.5	3968.7

72.0 gm. The subjects were then divided into 4 groups according to the quartile range in which they fell.

The mean values for dietary fat, milk fat, lipase, esterase, acid phosphatase and alkaline phosphatase contents of milk for these 4 different groups are given in table 1.

DISCUSSION

It can be seen from table 1 that progressive increases in fat content of milk are observed from group 1 to group 3, no further increase being observed between group 3 and group 4. The product moment correlation between dietary fat and the fat content of milk was also estimated (from the original data) and found to be 0.7932, a high value. It can also be seen from table 1 that lipase, esterase and alkaline phosphatase activities increase, and that acid phosphatase activity decreases, with increase in the fat content of milk. The correlation coefficients between the fat content of milk on the one hand and concentrations of lipase, esterase and alkaline phosphatase on the other hand are respectively 0.3940, 0.8604, and 0.8548. It will be observed that they are all highly significant. On the contrary the correlation coefficient between the fat content of milk and acid phosphatase is not significant ($r = -0.1006$). It is also observed that there are no differences in lipase, esterase, acid phosphatase, and alkaline phosphatase activities between group 3 and group 4. This suggests that increase in dietary intake of fat up to a certain amount, to be determined, increases the fat content of milk as well as its lipase, esterase and alkaline phosphatase activities. It is relevant to mention in this connection that neither dietary protein nor milk protein has been found to affect the activities of these enzymes.[2] This suggests a definite relation between dietary fat, milk fat and the lipase, esterase and phosphatase contents of milk. Thus it would appear that increase in dietary fat up to a certain point may not only increase the fat content of milk but also enrich the milk with certain enzymes like lipase, esterase and alkaline phosphatase.

SUMMARY

Dietary fat, milk fat and the lipase, esterase, acid and alkaline phosphatase contents of milk of 60 lactating women were estimated and it was found that up to a certain limit increases in dietary fat were accompanied by increases in milk fat as well as in lipase, esterase, and alkaline phosphatase contents of milk.

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LITERATURE CITED

Arshavskii, A. 1940 Lipase of mother's milk and its importance in regard to the disadvantages of bottle feeding. Pediatriya, *4:* 11.

Banerjee, S., and D. K. Biswas 1957 Studies on nutritive value of the cooked diet consumed by students of Eden Hindu Hostel, Calcutta. Ind. Jour. Med. Res., *45:* 411.

Boissonnas, R. A. 1948 Esterases. I. The measurement of pancreatic (hog) lipase activity. Helv. Chim. Acta, *31:* 1571.

Chanda, E., and E. C. Owen 1951 The composition of human milk with special reference to the relation between phosphorus partition and phosphatase and to the partition of certain vitamins. Brit. J. Nutrition, 5: 228.

Chiba, K., T. Moriwaki, S. Suzuki, N. Hirai and K. Imahori 1956 Report on chemical study of human milk. Nat. Soc. Rept. of Ochanimizu Univ., *6:* 224.

Fiske, C. H., and Y. Subbarow 1925 Colorimetric determination of phosphorus. J. Biol. Chem., 66: 375.

Harrer, C. J., and C. G. King 1941 Ascorbic acid deficiency and enzyme activity in guinea-pig tissues. Ibid., *138:* 111.

Jacqmain, D., and M. Loncin 1953 The lipolytic phenomena in milk. Intern. Congr. Proc., 13th Congr., 2: 368.

Karmarkar, M. G., A. D. Deodhar, J. Kapur and C. V. Ramakrishnan 1958 Effect of dietary fat on fat content of milk. Lancet, 2: 909.

Karmarkar, M. G., J. Kapur, A. D. Deodhar and C. V. Ramakrishnan 1959 Studies on human lactation. Ind. Jour. Med. Res., *47:* 344.

Morton, R. K. 1955 Phosphomonoesterase of milk. In: Methods in Enzymology. Edited by S. P. Colowick and N. O. Kaplan, Acad. Press Inc., New York, Vol. II, p. 427.

Stewart, R. A., E. Platou and V. J. Kelly 1958 The alkaline phosphatase content of human milk. J. Biol. Chem., 232: 777.

Vittu, C. 1946 The phosphomonoesterase system of human milk and its sensitivity to the action of sulfanilamide. C. R. Soc. Biol., *140:* 225.

[2] Authors' unpublished observations.

Studies on Unidentified Chick Growth Factors Apparently Organic in Nature

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Morrison and associates ('55, '56), Dannenburg and associates ('55), and Camp, Reid and Couch ('55) reported that, when a mixture of crude sources of unidentified growth factors was fed to chicks, the observed growth increases appeared to be due to the presence in these materials of both unidentified organic and inorganic constituents. The evidence for the existence of growth-stimulating substances organic in nature was based upon the markedly greater growth which occurred in chicks when the diet contained the intact supplements in comparison with that obtained with an equivalent amount of mineral matter produced by ashing at temperatures of 525 to 700°C. The procedure of including in the diet a quantity of the ash of unidentified factor supplements equivalent to that present in the amount used to stimulate chick growth has been followed in further work on unidentified chick growth factors apparently organic in nature. The results of this work are presented in this report.

EXPERIMENTAL

Purebred White Plymouth Rock chicks or crossbred chicks of White Plymouth Rock hens mated to Vantress males were used in this investigation. With the exception of one experiment the chicks were hatched from the eggs of hens maintained at the Cornell University poultry plant. The hens were fed a simplified diet composed largely of corn and soybean meal. No sources of unidentified chick growth factors were included in the diet. The hens were housed in pens with raised wire-mesh floors in order to prevent coprophagy.

Duplicate lots of 13 to 20 chicks per lot were subjected to each treatment. A uniform number of chicks per lot was used in each experiment. In 7 of the 12 experiments reported in this paper, each lot contained an equal number of male and female chicks. In the remaining experiments, only male chicks were used. The chicks were placed on experiment at approximately one day of age. The duration of all experiments was 4 weeks.

The chicks were housed in galvanized metal batteries with raised wire-mesh floors, equipped with automatically-controlled electrical heating units. The chicks of each lot were weighed as a group at the start of the experiments and individually every week thereafter. Each chick was identified with a numbered wingband. Feed and water were supplied ad libitum. A weekly record of feed consumption was made at the time the chicks were weighed.

Two basal diets designated diets A and B were used in all experiments except experiment 1 in which diet AA was fed. This diet was identical with diet A except that it contained 5% less soybean protein and 5% more glucose. The composition of diets A and B is given in table 1. The metabolizable energy content of diet A was 3065 Cal./kg and that of diet B, 3495. The protein content (nitrogen × 6.25) of these diets was 27.1 and 30 1%, respectively. Both diets contained an excess amount of protein in relation to energy content. Because of higher energy content, the methionine, glycine, mineral and vitamin additions to diet B were increased so as to make the intake of these materials per chick per day approximately the same as those of the chicks fed diet A. An antioxidant was included in diet B in order to prevent the corn oil from becoming rancid during the course of the experiments.

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TABLE 1

Composition of basal diets

Ingredient	Amount	
	Basal A	Basal B
	%	%
Glucose monohydrate[1]	55.55	45.21
Soybean protein[2]	30.00	33.38
Hydrogenated oil[3]	3.00	—
Corn oil[4]	—	10.00
Cellulose[5]	3.00	3.23
DL-Methionine	0.70	0.75
Glycine	0.30	0.32
Mineral mixture[6]	5.43	5.84
Ash unidentified growth factors (UGF)	1.30	—
Vitamin mixture	0.72[7]	1.25[8]
2,6-Ditertiary butyl-4-methyl phenol (BHT)	—	0.022

[1] Cerelose.
[2] Drackett Assay C-1.
[3] Hydora.
[4] Mazola.
[5] Solka Floc.
[6] The grams per 1000 gm of Analytical Reagent Grade chemicals in the mineral mixture were as follows: 396.1 $CaHPO_4$, 274.8 $CaCO_3$, 159.7 KH_2PO_4, 110.5 NaCl, 46.0 $MgSO_4$, 6.1 $FeSO_4 \cdot 7H_2O$, 6.1 $MnSO_4 \cdot H_2O$, 0.31 $CuSO_4 \cdot 5H_2O$, 0.18 $ZnCl_2$, 0.15 $Na_2MoO_4 \cdot 2H_2O$, 0.048 KI, 0.031 $CoCl_2 \cdot 6H_2O$.
[7] The grams and units per 1000 gm of vitamins and other substances in the vitamin mixture were as follows: 208.3 choline chloride, 34.7 inositol, 6.9 niacin, 2.8 *d*-calcium pantothenate, 2.8 α-tocopheryl acetate, 1.4 thiamine·HCl, 1.4 riboflavin, 0.63 pyridoxine·HCl, 0.56 folic acid, 0.069 menadione, 0.028 biotin, 0.0028 vitamin B_{12}, 6,940 IU vitamin A, 530 ICU vitamin D_3, 740.3 vitamins A, D_3 and E diluents and glucose.
[8] The grams and units per 1000 gm of vitamins and other substances in the vitamin mixture were as follows: 129.8 choline chloride, 21.6 inositol, 5.7 α-tocopheryl acetate, 4.3 niacin, 3.5 *d*-calcium pantothenate, 0.87 thiamine·HCl, 0.87 riboflavin, 0.39 pyridoxine·HCl, 0.35 folic acid, 0.083 menadione sodium bisulfite, 0.017 biotin, 0.0035 vitamin B_{12}, 4,330 IU vitamin A, 865 ICU vitamin D_3, 1.9 diphenyl-*p*-phenylenediamine (DPPD), 830.6 vitamin A, D and E diluents and glucose.

A mixture of crude materials, hereafter designated UGF, and a liver extract were used as sources of unidentified chick growth factors. The former was composed of 50% distillers' dried solubles, 25% fish solubles, and 25% dried whey product. The ash included in diets A and AA was obtained by burning the UGF at 525°C until a light gray product was obtained. The percentage of ash given in diet A, table 1, is the average quantity equivalent to 12% UGF.

The UGF and liver extract[1] used in the experiments were obtained from the same original supplies. When these unidentified factor supplements were included in the basal diet, appropriate adjustments were made so as to maintain the protein content of the diets constant.

The UGF contained 69.4 mg of zinc per kg, and the liver fraction 29.4 mg per kg. The quantities of potassium in the UGF and the liver fraction were 2.1 and 2.93% respectively, and the quantities of sodium were 0.91 and 0.88% respectively.

All values for zinc content presented in this report were determined according to the colorimetric procedure of the A.O.A.C. ('55). The values for potassium and sodium were determined by the procedure described by Mathis ('56).

The results of the experiments were subjected to analysis of variance according to Snedecor ('56). Afterwards, Duncan's multiple range test described by Federer ('55) was applied to the results where necessary in order to locate the sites of significant growth differences.

RESULTS

The first group of experiments was conducted to determine if the intact UGF pro-

[1] The liver extract was Liver Fraction No. 1 of Wilson, Laboratories, Wilson and Company, Chicago, Illinois.

TABLE 2

Effect of crude sources of unidentified factors (UGF) on chick growth

Experiment	Av. weight at 4 weeks		Gain over basal	Gain/feed	
	Basal A	UGF		Basal	UGF
	gm	*gm*	%		
1	337(36)[1]	431(35)	31.7	0.52	0.58
2	339(36)	380(39)	13.7	0.56	0.60
3	360(39)	432(40)	22.4	0.55	0.63
4	344(35)	411(36)	22.0	0.63	0.58
5	322(35)	382(38)	21.4	0.46	0.56
Average	340(210)	407(217)	22.3	0.55	0.59

[1] Survivors; 40 chicks per treatment at start.

moted greater growth in chicks than that observed when the basal diet, containing an equivalent amount of ash from the mixture, was fed. The results of the experimental work are presented in table 2. The intact UGF was found to promote markedly greater growth than that promoted by the basal diet containing the ash. The average increase in gain was 22.3% and the range in gain in the experiments varied from 13.7% to 31.7%. The growth increases were found to be highly significant ($P < 0.01$). The unit gain per unit of feed consumed was also improved to the extent of 7.3%.

O'Dell, Newberne and Savage ('58) pointed out that the basal diets used in the experiments of Morrison and associates ('55, '56) and Dannenburg and associates ('55) were probably deficient in zinc and perhaps potassium. However, by including the ash of an equivalent amount of the intact UGF in the diet the potassium content was increased from 0.25 to 0.52%. This quantity has been found by Leach and associates ('59) to be more than adequate for maximum growth of chicks under the experimental conditions. The amount of zinc added to the basal diet by means of the ash was 8.3 mg/kg. Since the basal diets contained 11.4 ± 3.1 mg of zinc per kg, the ash addition increased the total zinc content of the diets to approximately 19.7 mg/kg. This amount has been found by Zeigler, Leach and Norris ('58) to be sufficient for maximum growth of chicks maintained in a zinc-free environment and fed a diet containing casein but inadequate when the diet contained soybean protein. The increased growth obtained with the intact UGF, however, was not due to zinc functioning independently, since the quantity of zinc supplied by it was no greater than that supplied by the ash.

The possibility that the improved growth was caused by the presence of minerals in the intact UGF, which were sublimed by ashing at a temperature of 525°C, appears ruled out except for selenium. The basal diet was found to contain an adequate quantity of selenium.[2] No evidence exists for a requirement for other mineral elements which sublime at this temperature. The results of the experiments showed, therefore, that the UGF contains an unidentified organic factor or factors required directly for chick growth or which promotes chick growth indirectly by rendering the zinc in the mixture in some unexplained manner more available to the chick.

On completion of these experiments studies were initiated on the unidentified growth factor reported to be present in liver and liver extracts by Edwards and associates ('55) and others. Further work was also conducted on the unidentified growth factors in the UGF. Basal diet B was fed in these studies. This diet was subsequently shown by Leach and associates ('59) to be deficient in potassium, and by Zeigler, Leach and Norris ('58) to be deficient in zinc when the chicks were reared in a zinc-free environment.

The results of two experiments are summarized in the first section of table 3 and show that the addition of 1.5% of liver extract to basal diet B promoted a striking increase in growth. The growth increase was found to be highly significant ($P < 0.01$).

[2] Unpublished results, Leach, R. M., Jr., and L. C. Norris, 1959.

TABLE 3

Interrelationship of unidentified liver-extract factor and zinc

Treatment	Av. weight at 4 weeks	Gain/feed
	gm	
Basal diet B		
Basal (6, 7)[1]	371(59)[2]	0.63
+ 1.5% liver extract (LE)(6, 7)	469(62)	0.66
+ ash = 1.5% LE (7)	380(30)	0.63
+ ash UGF (7)	457(32)	0.65
+ ash UGF + 1.5% LE (7)	532(31)	0.68
+ UGF (6, 7)	524(63)	0.68
Basal diet B with adequate zinc		
Basal (8, 9)	522(53)[3]	0.71
+ 2% liver extract (LE) (8, 9)	576(55)	0.71
+ 0.23% K (9, 10, 11, 12)	571(115)	0.74
+ 0.23% K + 2% LE (9, 10, 11, 12)	588(114)	0.73
+ 0.23% K + UGF (12)	647(32)	0.76

[1] Experiment.
[2] Survivors; 64, 64, 32, 32, 32 and 64 chicks per treatment, respectively, at start.
[3] Survivors; 56, 56, 118, 118 and 32 chicks per treatment, respectively, at start.

In contrast an amount of liver ash equivalent to that in the intact extract failed to stimulate a significant growth increase. When the ash of the UGF was included in the basal diet in an amount equivalent to the quantity of the intact material supplied in the previous group of experiments, a significant growth increase ($P < 0.01$) occurred which was approximately equal to that obtained with the intact liver extract. A still further growth increase was obtained by additions to the basal diet of the intact UGF or a combination of UGF ash and liver extract. This growth improvement was also found to be significant ($P < 0.05$). The ash of the liver extract raised the potassium content of basal diet B from 0.27 to 0.31% and made the diet almost adequate in potassium, while the UGF ash increased the potassium content to 0.52%.

The liver extract ash, however, appeared to lack a substance present in the intact extract and in the UGF ash. The liver extract increased the zinc content of the basal diet 0.44 mg/kg, whereas the UGF ash increased it 6.9 mg/kg. The former quantity of zinc, according to Zeigler and Norris[3] would have no measurable effect on chick growth. On the other hand, the latter quantity would have a marked effect but would not entirely satisfy the zinc requirement of chicks fed a purified diet containing soybean protein. The results suggested, therefore, that the UGF ash stimulated chick growth by increasing the dietary zinc and that the liver extract and the UGF stimulated chick growth because of the presence of unidentified organic substances.

This finding was confirmed by the results of the work with liver extract presented in the second section of table 3. Basal diet B, supplemented with 50 mg zinc per kg, an amount more than sufficient to meet the chick's requirement for this mineral, according to Zeigler, Leach and Norris ('58), was used in these studies. When this diet was supplemented with liver extract an increase in growth was obtained which was significant ($P < 0.05$). Adding 0.23% potassium to the diet promoted a growth improvement approximately equal to that obtained with the liver extract. When both potassium and liver extract were fed, the increase in growth was little greater than that obtained when either one of these materials was fed alone. These results indicated that the growth-promoting effect of liver extract in these experiments was caused by its potassium content. The inclusion of zinc in the basal diet, therefore, appeared to replace the unidentified growth-promoting substance in the intact liver extract. This suggests the possibility that the un-

[3] Unpublished results, 1959.

TABLE 4

Need for unidentified factors by chicks supplied adequate quantities of zinc and potassium in the basal diet

Experiment	Av. weight at 4 weeks		Gain over basal	Gain/feed	
	Basal B	UGF[1]		Basal	UGF
	gm	*gm*	%		
9	586(24)[2]	610(26)	4.38	0.74	0.76
10	571(32)	647(32)	14.28	0.76	0.75
11	564(30)	596(30)	6.12	0.74	0.72
13	579(30)	635(30)	10.37	0.80	0.80
Average	575(116)	622(118)	8.77	0.76	0.76

[1] Distillers' dried solubles used as UGF in experiment 13.
[2] Survivors; 26, 32, 30 and 30 chicks per treatment, respectively, at start.

known organic substance in liver extract acted as a chelating agent, and thereby made the dietary zinc more available. Whether or not the UGF also contained the substance could not be determined from the evidence. Kratzer[4] has observed that zinc functions as a partial substitute for the unidentified, growth-promoting properties of a soybean meal extract. In addition Kratzer and associates ('59) have obtained evidence that EDTA (ethylenediaminetetraacetic acid),[5] a chelating agent, appears to make the zinc in a purified diet containing soybean protein more available.

Although excellent growth was promoted in the chicks by supplying a diet adequate in both zinc and potassium, a still further growth increase was obtained in repeated experiments by supplementing the diet with sources of unidentified chick growth factors. This growth increase was found to be highly significant ($P < 0.01$). The UGF previously described, and distillers' dried solubles[6] were used to provide unidentified growth factors in the experiments.

The results of the experiments are presented in table 4. The average gain in weight promoted by the sources of unidentified growth factors was 8.55% and the range in gain varied from 4.38 to 14.28. The cause of this variation is not clear at present, but may be related to variation in the composition of some of the ingredients used in the basal diet. In work on the growth-stimulating properties of vegetable fats by Dam and associates ('59), evidence of an interaction between the level of corn oil in the basal diet and the UGF was observed, indicating that at times corn oil exerted a sparing effect on the unidentified factors. In spite of the variability in results evidence of the existence of an organic, chick growth-promoting substance in sources of unidentified growth factors not identical with the one in liver extract has been obtained.

SUMMARY

Further evidence has been obtained of the presence, in crude materials, of unidentified substances, apparently organic in nature, which stimulate growth of chicks fed a purified diet containing soybean protein and adequate quantities of all known vitamins. One of these, found in liver extract, appeared to replace zinc to a great extent or to render it more available. Another factor(s), present in a mixture of distillers' dried solubles, dried whey and fish solubles, promoted growth of chicks fed a purified diet adequate in zinc.

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[4] Kratzer, F. W., University of California, Davis, 1959, personal communication to the authors.
[5] Versene.
[6] Supplied by J. A. Wakelam, The Distillers Company, Ltd., London, England.

LITERATURE CITED

Association of Official Agricultural Chemists 1955 Official Methods of Analysis, 8th ed., chap. 24, p. 453.

Camp, A. A., B. L. Reid and J. R. Couch 1956 Growth promoting activity of ash when fed in practical diets to chicks. Poultry Sci., 35: 621.

Dam, R., R. M. Leach, Jr., T. S. Nelson, L. C. Norris and F. W. Hill 1959 Studies on the effect of quantity and type of fat on chick growth. J. Nutrition, 68: 615.

Dannenburg, W. N., B. L. Reid, E. E. Rozacky and J. R. Couch 1955 An inorganic chick growth response. Poultry Sci., 34: 1023.

Edwards, H. M., Jr., D. Chin, L. C. Norris and G. F. Heuser 1955 Differentiation of unidentified chick growth factors in liver. J. Nutrition, 57: 329.

Federer, W. T. 1955 Experimental Design. Theory and Application. The Macmillan Company, New York, N. Y., chap. 2, p. 26.

Kratzer, F. H., J. B. Allred, P. N. Davis, B. J. Marshall and P. Vohra 1959 The effect of autoclaving soybean protein and the addition of ethylenediaminetetraacetic acid on the biological availability of dietary zinc for the turkey poult. J. Nutrition, 68: 313.

Leach, R. M., Jr., R. Dam, T. R. Zeigler and L. C. Norris 1959 The effect of protein and energy on the potassium requirement of the chick. Ibid., 68: 89.

Mathis, W. T. 1956 Report on the flame photometric determination of potassium and sodium in plant tissue. J. Assoc. Official Agr. Chem., 39: 419.

Morrison, A. B., R. Dam, L. C. Norris and M. L. Scott 1956 Further evidence on the requirement of the chick for unidentified minerals. J. Nutrition, 60: 283.

Morrison, A. B., M. L. Scott and L. C. Norris 1955 Evidence for an unidentified mineral required by the chick. Poultry Sci., 34: 738.

O'Dell, B. L., P. M. Newberne and J. E. Savage 1958 Significance of dietary zinc for the growing chick. J. Nutrition, 65: 503.

Snedecor, G. W. 1956 Statistical Methods, 5th ed. Iowa State College Press, Ames, Iowa.

Zeigler, T. R., R. M. Leach, Jr. and L. C. Norris 1958 Zinc requirement of the chick. Federation Proc., 17: 498.

The Influence of Selected Vegetable Fats on Plasma Lipid Concentrations and Aortic Atheromatosis in Cholesterol-Fed and Diethylstilbestrol-Implanted Cockerels

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Groen ('52), Kinsell ('53), Ahrens ('54), Beveridge ('55), Bronte-Stewart ('56) and Dam ('59) and their associates have shown that the feeding of various plant and marine fats to men will lower the concentration of cholesterol in their serum or plasma. It has been suggested that this effect may be related to the source of fat (i.e., animal or plant, (Beveridge et al., '55)), its iodine value (Ahrens et al., '57), the specific types of unsaturation in the constituent fatty acids (Sinclair, '56), the chain length of the fatty acids, and the presence of other substances, such as β-sitosterol (Beveridge et al., '57). Other investigators (Opdyke and Ott, '54; Swell et al., '55; Aftergood et al., '56; Jones et al., '56; Kritchevsky et al., '56; Portman et al., '56; Hegsted et al., '57a,b) have compared the influence of various fats on cholesterol concentrations and on vascular lesions in chickens, rabbits, rats and monkeys with results which often have differed from one another and from results in man.

We present here a comparison of the influence of several vegetable fats on plasma cholesterol concentrations and degree of aortic atheromatosis in cholesterol-fed and in diethylstilbestrol-implanted cockerels. Animal fats were excluded from the study to avoid the possible effect of a substance in them which raises blood cholesterol concentrations, and the vegetable fats chosen all had similar saponification numbers, and thus similar average chain length. The fats studied were cacao butter, olive oil, cottonseed oil, corn oil, safflower oil, linseed oil, and in one experiment, tung oil. They ranged in iodine value from 35 to 180.

EXPERIMENTAL

Procedures. The details of the experimental procedures and the composition of the basal ration have been described previously (Tennent et al., '57). For each experiment 60 8-week-old White Leghorn cockerels were set out in groups of 10 in wire batteries and fed experimental rations containing corn meal, soybean meal, fish meal, alfalfa meal, vitamins, minerals, and vegetable fat for 8 weeks; each group receiving a given fat for the entire period. The fats were fed as 5% of the diet, replacing an equal weight of corn meal in the basal ration. The cholesterol-containing ration was made up by further substituting 2% U.S.P. cholesterol for an equal weight of corn meal. Butylated hydroxytoluene, 0.025%, was added to all diets to inhibit oxidation. The diets were freshly made up each week and the stocks were kept in tightly closed cans in the cold room.

The total calculated neutral fat content of the cholesterol-containing diets was 8.21%; 5% from the added fat, and 3.21% from the corn meal, soybean meal, and fish meal in the ration. Of the total calories, 22% were derived from fat. Table 1 presents the calculated fatty acid composition of the total neutral fat in the several rations used.

The experiments were terminated after 8 weeks. Food was withheld overnight, 4 ml of blood were drawn from the alar vein and mixed with citrate (ACD) solution, the birds were decapitated, and the thoracic and brachiocephalic arteries were re-

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TABLE 1

Fatty acid composition and iodine number of total neutral fat in rations fortified with 5% fat and 2% cholesterol[1]

Added fat	Saturated	Monoenoic	Dienoic	Polyenoic	Iodine number
	%	%	%	%	
Cacao butter	42.1	40.4	17.0	0.4	77.0
Olive oil	12.1	67.4	20.2	0.4	104.4
Cottonseed oil	21.6	32.0	46.1	0.4	119.6
Corn oil	15.2	36.1	48.3	0.4	128.8
Safflower oil	9.7	33.0	56.8	0.5	140.9
Linseed oil	12.6	29.7	26.5	31.2	162.3

[1] For all fats except corn oil, these values are calculated from data published by E. F. Drew & Co., Inc., Boonton, New Jersey.

TABLE 2

Plasma lipid concentrations, lesion scores, and weight gains in groups of 10 cholesterol-fed cockerels given different vegetable fats in the diet

	Plasma cholesterol	Lipid phosphorus	C/PL[1]	Lesion score	Weight gain
	mg %	*mg %*			*gm*
		First experiment			
Cacao butter	330	6.90	2.00	1.45	949
Olive oil	238	5.51	1.82	0.80	903
Cottonseed oil	339	6.45	2.19	1.40	867
Safflower oil	208	5.21	1.65	1.00	879
Linseed oil	235	5.47	1.75	0.51	855
Tung oil	237	4.85	2.05	1.07	560
		Second experiment			
Cacao butter	275	6.75	1.73	1.43	919
Olive oil	233	5.67	1.72	1.31	930
Cottonseed oil	367	7.46	2.13	1.95	941
Corn oil	301	5.81	2.15	1.95	959
Safflower oil	178	5.70	1.35	1.35	910
Linseed oil	171	4.98	1.40	0.83	953
		Third experiment			
Cacao butter	256	5.35	1.98	1.25	928
Olive oil	331	6.03	2.23	1.40	906
Cottonseed oil	299	6.36	1.93	1.70	919
Corn oil	189	4.56	1.69	0.75	904
Safflower oil	179	4.62	1.60	0.85	880
Linseed oil	210	4.81	1.75	1.30	890
		Fourth experiment			
Cacao butter	283	6.23	1.87	1.10	995
Olive oil	318	6.09	2.13	1.50	1020
Cottonseed oil	305	6.47	1.90	1.67	964
Corn oil	235	5.76	1.71	1.70	1067
Safflower oil	239	5.94	1.79	1.40	1043
Linseed oil	225	5.27	1.73	1.30	1000
		Summary			
Cacao butter	285	6.28	1.90	1.31	948
Olive oil	276	5.82	1.98	1.25	940
Cottonseed oil	326	6.67	2.04	1.68	923
Corn oil	237	5.35	1.85	1.47	977
Safflower oil	200	5.24	1.60	1.15	928
Linseed oil	209	5.13	1.66	0.99	925

[1] Cholesterol/phospholipid ratio.

TABLE 3

Plasma lipid concentrations, lesion scores, and weight gains in groups of 10 estrogen-implanted cockerels given different vegetable fats in the diet

	Plasma cholesterol	Lipid phosphorus	C/PL[1]	Lesion score	Weight gain
	mg %	*mg %*			*gm*
		First experiment			
Cacao butter	811	128.5	0.26	0.83	923
Olive oil	710	118.1	0.24	0.95	977
Cottonseed oil	860	146.2	0.24	0.67	1029
Corn oil	816	153.2	0.21	0.76	1041
Safflower oil	782	124.8	0.26	0.85	984
Linseed oil	734	134.3	0.22	0.92	1013
		Second experiment			
Cacao butter	548	87.1	0.25	0.85	971
Olive oil	462	64.6	0.30	1.10	982
Cottonseed oil	703	110.6	0.26	1.20	1035
Corn oil	303	47.5	0.26	0.94	957
Safflower oil	439	71.0	0.26	1.10	995
Linseed oil	516	74.3	0.30	1.20	1020
		Third experiment			
Cacao butter	633	108.4	0.24	0.62	1028
Olive oil	486	77.9	0.25	0.55	1087
Cottonseed oil	560	92.0	0.25	0.25	1167
Corn oil	556	99.1	0.23	0.33	1082
Safflower oil	520	99.2	0.21	0.30	1085
Linseed oil	534	99.5	0.22	0.10	1092
		Summary			
Cacao butter	655	106.7	0.25	0.77	974
Olive oil	542	84.1	0.26	0.87	1015
Cottonseed oil	697	114.3	0.25	0.71	1077
Corn oil	516	89.8	0.23	0.68	1027
Safflower oil	563	95.7	0.25	0.75	1021
Linseed oil	587	99.8	0.25	0.74	1042

[1] Cholesterol/phospholipid ratio.

moved and graded for degree of atheromatosis, using a scale of 0 to 4. Plasma aliquots were analyzed for total cholesterol (Abell et al., '52) and for lipid phosphorus (King, '51; Zilversmit and Davis, '50).

Results. In 4 experiments, the cholesterol-containing ration was fed with the results shown in table 2. Tung oil was used instead of corn oil in the first experiment.

In three experiments, cockerels were implanted subcutaneously with diethylstilbestrol, 15 mg every 10 days, with the results shown in table 3. The diets of these birds contained added fat, *but no added cholesterol.*

DISCUSSION

From the data in table 2 it is apparent that the nature of the fat in the diet of cholesterol-fed cockerels influenced their plasma lipid concentrations. The differences in plasma cholesterol, plasma lipid phosphorus, and cholesterol/phospholipid ratio among the groups fed the different oils may be expected to occur by chance less than once in a thousand times. Cottonseed oil, in spite of its 50% linoleic acid content, uniformly produced high plasma lipid concentrations. Cacao butter and olive oil also produced high concentrations, while linseed and safflower oils produced low lipid values.

The effect of the various fats on plasma cholesterol level was not related to the amount of any single fatty acid constituent, and was only poorly related to iodine number. A satisfactory relation was derived, however, following the lead of Keys, Anderson and Grande ('57), by pooling into a single term all fatty acids with more

than one double bond, and solving the equation

$$E_{cs} = a + b\,S + d\,P$$

where E_{cs} is plasma cholesterol concentration, S is the amount of saturated acid, P is the sum of the amounts of dienoic and polyenoic acids in the diet (table 1), and a, b, and d are constants.[1]

The average plasma cholesterol concentrations of cholesterol-fed birds given the various oils, with the exception of cottonseed oil, were described by the expression

$$E_{cs} = 313 + 0.15\,S - 1.84\,P$$

From this it follows that birds fed a diet containing 2% cholesterol and 8.21% neutral fat would have average plasma cholesterol concentrations of 327 mg % if the fat were completely saturated, 313 mg % if it were 100% monoenoic fat, and 129 mg % if it were 100% polyenoic fat. It is evident that the effects of saturated fat and of monoenoic fat could not be distinguished from one another, and that the plasma cholesterol concentration could be predicted from the polyunsaturated fatty acid content alone, the expression being

$$E_{cs} = 318 - 1.90\,P$$

This relation is illustrated in figure 1. It does not indicate whether polyunsaturated fats are "good," or saturated and monoenoic fats are "bad." It may be that one is acting as a diluent for the other.

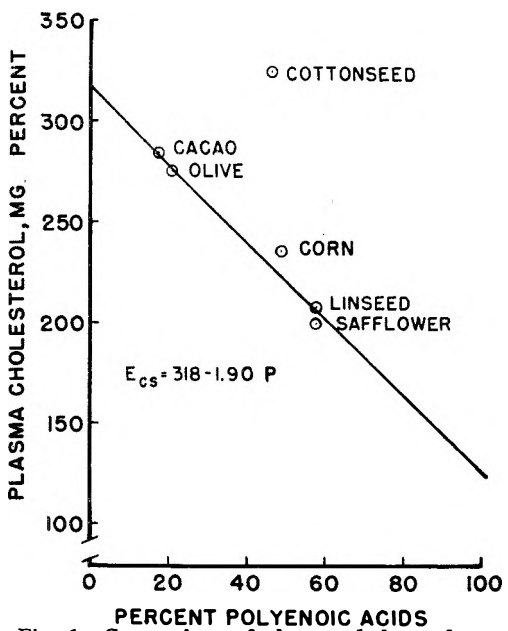

Fig. 1 Comparison of plasma cholesterol concentrations with per cent polyenoic acids in the dietary fat of cholesterol-fed cockerels.

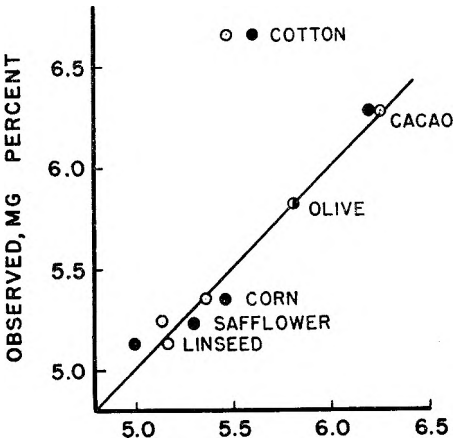

Fig. 2 Comparison of plasma lipid phosphorus concentrations observed in cholesterol-fed cockerels with those predicted from the regression equation, $E_{LP} = 6.02 + 0.0129S - 0.0173P$ (open circles), and from iodine number, $E_{LP} = 7.19 - 0.0141J$ (closed circles).

The predicted cholesterol concentrations all fall within 5% of the experimentally determined values, except in birds fed cottonseed oil, where the deviation is 30%. Plasma cholesterol concentrations in birds fed cottonseed oil were as high as predicted concentrations in birds fed a pure saturated fat.

Plasma lipid phosphorus concentration in cholesterol-fed birds could be predicted in similar fashion, the best line being described by the equation

$$E_{LP} = 6.02 + 0.0129S - 0.0173P$$

Birds fed a diet containing 2% cholesterol and 8.21% neutral fat would have average plasma lipid phosphorus of 7.31 mg % if the dietary fat were completely saturated, 6.02 mg % if it were 100% monoenoic, and 4.29 mg % if it were 100% polyenoic. Thus lipid phosphorus concentrations seem to be related to general unsaturation. As shown in figure 2, agreement with the de-

[1] A 4th term, cM, representing the influence of monoenoic acid, is omitted here. Its value is implicit in that $S + M + P = 100\%$ and the experiments were conducted at constant fat content. The omission is necessary for solution of the simultaneous equations.

rived equation is a little better than with values predicted on the basis of iodine number, using the equation

$$E_{LP} = 7.19 - 0.0141J$$

The nature of the fat in the diet had less influence on lesion score of cholesterol-fed birds than it had on their plasma lipid pattern. Cottonseed oil consistently produced a high lesion score, but results with the other 5 oils did not differ sufficiently to indicate a trend on the basis of the data at hand. Thus, the close connection between dietary fat and lipid pattern does not extend to lesion score.

We have previously seen a correlation between lipid pattern and lesion score in birds fed cholesterol and cottonseed oil (Tennent et al., '57). The correlation of lipid pattern with lesion score within groups for all of the fats here (table 4) was slightly better than that previously seen, and thus, among birds fed any given fat, those with the greatest plasma lipid concentrations also tended to be those with the greatest lesion score. Comparison of results with birds fed different fats, however, suggests that high plasma cholesterol concentrations in birds fed some fats, such as olive oil or cacao butter, may be less injurious than the same concentrations are in birds fed other fats, such as safflower or corn oils ($P = 0.1$).

Cottonseed oil had unexpected ability to raise lipid concentrations and lesion scores in these experiments, indicating that it must contain one or more specific substances with this action which are not present to great degree in the other fats studied. Opdyke and Ott ('54) have observed that different preparations of cottonseed oil differ in this regard. Jones, Reiss and Huffman ('56) have observed that cottonseed oil produces higher serum cholesterol concentrations in cholesterol-fed chickens than does corn oil. Thus the presence of certain substances of unknown identity in a vegetable fat may cause it to have an effect on lipid patterns of cholesterol-fed birds other than that predicted on the basis of fatty acid composition.

TABLE 4

Correlation (r) and regression (b) coefficients within groups of lesion scores on plasma lipid concentrations in cholesterol-fed cockerels

	r	b
Log plasma cholesterol, mg/100 ml	0.70	2.96
Log lipid phosphorus, mg/100 ml	0.59	4.56
Cholesterol/phospholipid ratio	0.64	1.15

Cockerels implanted with diethylstilbestrol (Chaikoff et al., '48) exhibit increased plasma cholesterol concentrations of endogenous origin and greatly increased plasma lipid phosphorus concentrations. Aortic lesions develop more slowly than in cholesterol-fed birds. In our experiments with diethylstilbestrol-implanted cockerels, the nature of the fat in the diet had no significant influence on lesion scores or plasma lipid concentrations. Thus the influence of the dietary fat on lipid pattern depends upon the mechanism by which the plasma lipids have been elevated.

SUMMARY

In cockerels fed diets containing 8.2% of vegetable fat and 2% of cholesterol, there was a close connection between plasma lipid pattern and the composition of the fat fed. Plasma cholesterol concentrations were inversely proportional to the percentage of polyenoic acids in the fat (or directly proportional to saturated plus monoenoic). Plasma lipid phosphorus concentrations were inversely proportional to the total amount of unsaturation. Aortic lesion scores were not closely related to the composition of the fat fed. It appears that a given plasma cholesterol concentration in birds fed some fats may not be so injurious as the same concentration would be in birds fed other fats.

The cottonseed oil used here had peculiarly iniquitous properties, producing high lesion scores and plasma lipid concentrations. Thus certain substances of unknown identity in a fat may cause it to have an effect on lipid patterns of cholesterol-fed birds other than that predicted on the basis of fatty acid composition.

High plasma lipid concentrations of endogenous origin, produced by implanting pellets of diethylstilbestrol, were not greatly influenced by the composition of the fat in the diet, and thus the influence of dietary fat on plasma lipid patterns depends upon the mechanism by which plasma lipids have been elevated.

ACKNOWLEDGMENT

The composition of diet with added corn oil, as shown in table 1, was calculated from analysis of a sample of the oil used. The authors are greatly indebted to Dr. Jules Hirsch, Rockefeller Institute for Medical Research, for this analysis; also to Marion Campbell and Ann Van Iderstine for technical assistance in the study.

LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

Aftergood, L., R. B. Alfin-Slater and H. J. Deuel, Jr. 1956 Comparative effect of cottonseed oil and lard on cholesterol metabolism in rat. Federation Proc., *15:* 541.

Ahrens, E. H. Jr., D. H. Blankenhorn and T. T. Tsaltas 1954 Effect on human serum lipids of substituting plant for animal foods in the diet. Proc. Soc. Exp. Biol. Med., *86:* 872.

Ahrens, E. H. Jr., W. Insull, Jr., R. Blomstrand, J. Hirsch, T. T. Tsaltas and M. L. Peterson 1957 The influence of dietary fats on serum-lipid levels in man. Lancet, *1:* 543.

Beveridge, J. M. R., W. F. Connell, G. A. Mayer, J. B. Firstbrook and M. S. DeWolfe 1955 The effects of certain vegetable and animal fats on the plasma lipids of humans. J. Nutrition, *56:* 311.

Beveridge, J. M. R., W. F. Connell and G. A. Mayer 1957 The nature of the substances in dietary fat affecting the level of plasma cholesterol in humans. Canad. J. Biochem. Physiol., *35:* 257.

Bronte-Stewart, B., A. Antonis, L. Eales and J. F. Brock 1956 Effects of feeding different fats on serum-cholesterol level. Lancet, *1:* 521.

Chaikoff, I. L., S. Lindsay, F. W. Lorenz and C. Entenman 1948 Production of atheromatosis in the aorta of the bird by the administration of diethylstilbestrol. J. Exp. Med., *88:* 373.

Dam, H., G. Kristensen, G. K. Nielsen and E. Søndergaard 1959 Influence of dietary cholesterol, cod liver oil and linseed oil on cholesterol and polyenoic fatty acids in tissues from fasted and non-fasted chicks. Acta Physiol. Scand., *45:* 31.

Groen, J., B. K. Tjiong, C. E. Kamminga and A. F. Willebrands 1952 The influence of nutrition, individuality and some other factors including stress on the serum cholesterol. An experiment of nine months duration in sixty normal human volunteers. Voeding, *13:* 556.

Hegsted, D. M., S. B. Andrus, A. Gotsis and O. W. Portman 1957a The quantitative effects of cholesterol, cholic acid, and type of fat on serum cholesterol and vascular sudanophilia in the rat. J. Nutrition, *63:* 273.

Hegsted, D. M., A. Gotsis and F. J. Stare 1957b The effect of various fats upon experimental hypercholesteremia in the rat. Ibid., *63:* 377.

Jones, R. J., O. K. Reiss and S. Huffman 1956 Corn oil and hypercholesteremic response in the cholesterol-fed chick. Proc. Soc. Exp. Biol. Med., *93:* 88.

Keys, A., J. T. Anderson and F. Grande 1957 Prediction of serum-cholesterol responses of man to changes in fats in the diet. Lancet, *2:* 959.

King, E. J. 1951 Micro-analysis in Medical Biochemistry. J. & A. Churchill, London, p. 67.

Kinsell, L. W., G. D. Michaels, J. W. Partridge, L. A. Boling, H. E. Balch and G. C. Cochrane 1953 Effect upon serum cholesterol and phospholipids of diets containing large amounts of vegetable fat. J. Clin. Nutrition, *1:* 224.

Kritchevsky, D., A. W. Moyer, W. C. Tesar, R. F. J. McCandless, J. B. Logan, R. A. Brown and M. E. Englert 1956 Cholesterol vehicle in experimental atherosclerosis. II. Influence of unsaturation. Am. J. Physiol., *185:* 279.

Opdyke, D. F., and W. H. Ott 1954 Influence of source of cholesterol, grade of cottonseed oil, and breed on experimental avian atherosclerosis. Proc. Soc. Exp. Biol. Med., *85:* 414.

Portman, O. W., D. M. Hegsted, F. J. Stare, D. Bruno, R. Murphy and L. Sinisterra 1956 Effect of the level and type of dietary fat on the metabolism of cholesterol and beta lipoproteins in the cebus monkey. J. Exp. Med., *104:* 817.

Sinclair, H. M. 1956 Deficiency of essential fatty acids and atherosclerosis. Lancet, *1:* 381.

Swell, L., D. F. Flick, H. Field and C. R. Treadwell 1955 Role of fat and fatty acid in absorption of dietary cholesterol. Am. J. Physiol., *180:* 124.

Tennent, D. M., H. Siegel, G. W. Kuron, W. H. Ott and C. W. Mushett 1957 Lipid patterns and atherogenesis in cholesterol-fed chickens. Proc. Soc. Exp. Biol. Med., *96:* 679.

Zilversmit, D. E., and A. K. Davis 1950 Microdetermination of plasma phospholipids by trichloracetic acid precipitation. J. Lab. Clin. Med., *35:* 155.

Amino Acid Requirements for Maintenance in the Adult Rooster

II. THE REQUIREMENTS FOR GLUTAMIC ACID, HISTIDINE, LYSINE AND ARGININE[1]

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To date no values for the amino acid requirement for maintenance in the chicken are available. Ariyoshi ('57) has presented estimated values based on data obtained with surgically prepared roosters using whole egg protein as the nitrogen source. His data represent an approximation of the requirements since they are based on the assumption that the amino acid balance of whole egg protein is an "ideal" one because of its high biological value.

A previous report from this laboratory (Leveille and Fisher, '58) described the development of a free amino acid diet for adult roosters and its use in determining the minimal nitrogen and energy requirements for maintenance in protein depleted and non-depleted animals. In the present report data are presented on the quantitative requirements for L-glutamic acid, L-histidine, L-lysine and L-arginine for the maintenance of nitrogen equilibrium in the adult non-protein depleted rooster.

EXPERIMENTAL

White Leghorn roosters at least 12 months of age were used in all studies. The animals were maintained and standardized prior to receiving the test diets, as described by Leveille and Fisher ('58).

For the arginine studies only, the animals were force-fed as previously described. In all other experiments the roosters were given their diets in pelleted form and trained to consume their daily allotment within a 30-minute period.

The use of pelleted feed[2] made it possible to abandon the cumbersome force-feeding procedure since (1) feed wastage was negligible and (2) the animals readily consumed all the feed offered. It was found that the calculated metabolizable energy content of the pelleted diet could be reduced from 120 to 100 Cal./kg/day without resultant loss in body weight or change in nitrogen excretion. It is believed that the rate of passage of food may have been affected adversely in former experiments since considerable water had been added for force-feeding purposes. The bicarbonate which had been previously added was found to be unnecessary and was removed from the diet. The removal of the bicarbonate overcame the regurgitation difficulty previously described (Leveille and Fisher, '58). It would appear that the regurgitation was the result of pressure developed in the crop by the liberation of carbon dioxide.

The complete starting diets contained 13 amino acids in the proportions found in whole egg protein (Leveille and Fisher, '58) plus glycine to supply additional nitrogen such that when fed at the proper energy intake level all diets provided 280 mg N/kg/day. The composition of the starting diets used in the arginine study

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[2] A California Laboratory Pellet Mill was purchased from a special grant made available for this purpose by the National Science Foundation.

(diet A) and in other experiments (diet B) are presented in table 1.[3]

The experimental procedure employed consisted of feeding the animals the complete starting diet for a 5-day period and then each of the test diets with decreased amino acid increments for successive 5-day periods. The findings to be reported should not necessarily be extended beyond the experimental feeding period. The 5-day period used was based on the observation of Fritz et al. ('36) that a three-day period was sufficient to overcome the effect of the previous diet on nitrogen excretion in the adult rooster. Three or 4 birds were used in each experiment as indicated in the tables of results. Daily nitrogen balances were determined during the last three days of each period. Feeding and collection were carried out at 7 A.M. each morning thus insuring a complete 24-hour collection period. As the amount of a given amino acid was decreased it was replaced by an isonitrogenous amount of glycine or L-glutamic acid; the small differences resulting from this exchange were corrected by adding or removing dextrin. All nitrogen intake or excretion values in the tables are mean values for the number of birds indicated. Since nitrogen balance was carried out for three days on each treatment the nitrogen excretion values represent means of 9 or 12 values (three days × three or 4 birds).

TABLE 1

Composition of experimental diets

Ingredient	Amount	
	Diet A[1]	Diet B[2]
	%	%
L-Arginine·HCl	0.48	0.56
L-Histidine·HCl·H_2O	0.16	0.19
L-lysine·HCl (99%)	0.48	0.57
L-Tyrosine	0.25	0.30
L-Tryptophan	0.08	0.09
L-Phenylalanine	0.33	0.39
L-Cystine	0.14	0.16
DL-Methionine	0.23	0.27
DL-Threonine	0.48	0.57
L-Leucine	0.53	0.64
DL-Isoleucine	0.85	1.02
DL-Valine	0.82	0.97
L-Glutamic acid	1.45	1.72
Glycine	0.73	0.67
B vitamin mix[3]	0.15	0.15
Vitamin A, D, and E mix[3]	0.10	0.15
Choline chloride	0.20	0.24
Mineral mix[3]	3.00	3.58
Fiber	4.00	4.00
Corn oil	12.00	12.00
Antiacid adsorbent[3]	1.00	1.00
Starch	—	48.51
Cerelose	36.54	17.25
Dextrin	35.00	5.00
Sodium bicarbonate	0.50	—
Potassium bicarbonate	0.50	—
Totals	100.00	100.00

[1] Force-fed in arginine experiment at the rate of 31 gm/kg/day.

[2] Fed in all other experiments at the rate of 26 gm/kg/day.

[3] Fisher and Johnson ('56).

RESULTS

Two considerations were applied to the evaluation of a specific amino acid requirement: (1) the change in nitrogen excretion and (2) the change from a positive to a negative balance. Since all diets supplied the same calculated amount of nitrogen (280 mg/kg/day), nitrogen balance is merely a reflection of the nitrogen excretion; thus emphasis was placed on nitrogen excretion rather than nitrogen balance. Since Allison ('51) and others have shown that nitrogen equilibrium can be maintained on many intake levels the following differentiation was made in defining amino acid adequacy: The lowest level of an amino acid which would maintain the same level of nitrogen excretion observed on the starting diet (basal N excretion) is considered the *requirement* for that amino acid. The lowest level of an amino acid which still maintained the animal in positive nitrogen balance is considered the *minimal maintenance level.* Body weight changes were not considered indicative of dietary adequacy since the greatest change observed was less than 4% and in the majority of cases less than 1%. Such small variations are easily due to differences in water consumption and feather losses.

[3] In changing from diet A to diet B technical considerations of the pelleting process made it necessary to replace most of the dextrin by starch. As mentioned in the text, bicarbonate was omitted and the energy and amino acid levels were adjusted. To carry out the pelleting approximately 15% of cold water had to be mixed into the diet to permit proper binding in the absence of steam or heat compression which might have been deleterious. Immediately after pelleting the pellets were dried at 35 to 38°C for 12 to 24 hrs.

TABLE 2

The maintenance requirement for L-*glutamic acid and* L-*histidine in the adult rooster*

Amino acid fed	Body weight			N intake	N excretion	N balance
	Initial	Final	Δ			
mg/kg/day	*gm*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
			Glutamic acid			
222 (4)[1]	2342	2381	+39	265	238 ± 10[2]	+27
0 (4)	2381	2396	+15	262	243 ± 8	+19
			Histidine			
50 (4)	2679	2728	+49	263	216 ± 4	+47
0 (4)	2728	2750	+22	260	222 ± 5	+38

[1] Number of animals.
[2] Standard error of the mean.

TABLE 3

The maintenance requirement for L-*lysine in the adult rooster*

Lysine	Body weight			N intake	N excretion	N balance
	Initial	Final	Δ			
mg/kg/day	*gm*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
117 (4)[1]	2390	2396	+ 6	285	253 ± 5[2]	+32
29 (4)	2396	2384	−12	301	240 ± 6	+61
14 (4)	2384	2370	−14	299	260 ± 6	+39
6 (4)	2370	2355	−15	294	278 ± 7	+16
0 (4)	2355	2362	+ 7	294	273 ± 7	+21

[1] Number of animals.
[2] Standard error of the mean.

The results of the nitrogen balance studies are given in tables 2 through 4 and figures 1 and 2. The data presented in table 2 demonstrate the non-essentiality of glutamic acid and histidine for the maintenance of nitrogen balance in the adult rooster. It is evident that the complete absence of either of these amino acids from the diet had no effect on nitrogen excretion. Animals receiving diets deficient in either glutamic acid or histidine longer than the usual 5-day period (8 days) also did not show evidence of change in nitrogen excretion. Since no change in nitrogen excretion occurred with two such widely differing levels for each amino acid, further study of intermediate levels was not deemed necessary.

The requirement for lysine was found to be very low. The least squares line in figure 1 (data in table 3) show the requirement to be no greater than 29 mg/kg/day with no further improvement in nitrogen retention by increases in the lysine intake up to 117 mg/kg/day. Figure 1 also shows that the animals were not in negative nitrogen balance even in the complete absence of lysine from the diet. Nitrogen excretion in excess of 280 mg/kg/day would have been necessary for negative nitrogen balance. The nitrogen excretion, (table 3)

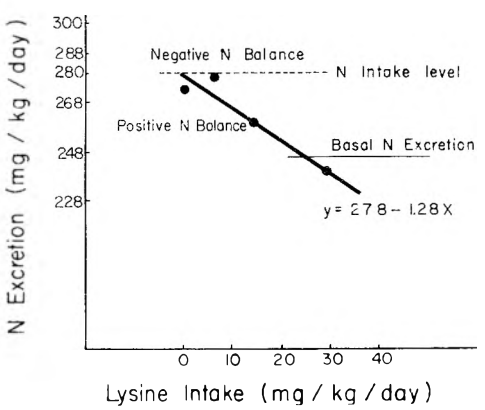

Fig. 1 The effect of lysine intake on nitrogen excretion in adult roosters receiving a free amino acid diet, mean values for 4 animals. Dotted line represents total N intake of 280 mg/kg/day and solid line represent a N excretion of 246 mg/kg/day based on the average values for the starting diet supplying 117 mg lysine and the diet containing the next highest level of lysine (29 mg/kg/day).

TABLE 4

The maintenance requirement for L-*arginine in the adult rooster*

Arginine	Body wt.			N intake	N excretion	N balance
	Initial	Final	Δ			
mg/kg/day	*gm*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
			Experiment 1			
121 (4)[1]	2264	2269	+ 5	274	226 ± 8[2]	+48
0 (4)	2269	2268	− 1	274	358 ± 6	−84
			Experiment 2			
121 (3)	2348	2319	− 29	280	238 ± 9	+42
62 (3)	2287	2302	+ 15	296	255 ± 6	+41
31 (3)	2305	2282	− 23	293	303 ± 18	−10
15 (3)	2277	2260	− 17	263	357 ± 17	−94
			Experiment 3			
62 (4)	2340	2335	− 5	270	264 ± 17	+ 6
46 (4)	2338	2328	− 10	299	312 ± 12	−13
62 (4)	2326	2320	− 5	285	253 ± 12	+32
			Experiment 4			
121 (4)	2308	2322	+ 14	286	230 ± 5	+56
100 (4)	2322	2320	− 2	298	228 ± 12	−70
80 (4)	2320	2321	+ 1	280	246 ± 6	+34
62 (4)	2321	2301	− 20	280	283 ± 9	− 3

[1] Number of animals.
[2] Standard error of the mean.

resulting from diets devoid of or supplying 6 mg/kg/day of lysine was 278 and 273 mg/kg/day respectively. A minimum level for the maintenance of nitrogen equilibrium could not be demonstrated.

For the rooster, in contrast to the adult human (Rose et al., '54) and rat (Burroughs et al., '40), arginine was found to be an important essential amino acid for maintenance. As can be seen from table 4 and figure 2 this requirement was found to be much greater than for any other amino acid studied to date. The nitrogen excretion was found to be a very sensitive measure of the arginine adequacy of the diet. Figure 2 indicates an inverse exponential relationship between arginine intake and nitrogen excretion ($Y = 385 - 2.436X + 0.009X^2$, where X and Y are nitrogen intake and nitrogen excretion in milligrams/kilograms/day respectively). The deviations of the observed nitrogen excretion values from the calculated curve are small and the standard error of the means was ± 4.03 mg N/kg/day. From figure 2 it is also evident that the level of arginine cannot be decreased below 120 mg/kg/day without a resultant increase in nitrogen excretion. This level is therefore considered the arginine requirement. However the minimal level for maintaining nitrogen balance (fig. 2) is 54 mg/kg/day.

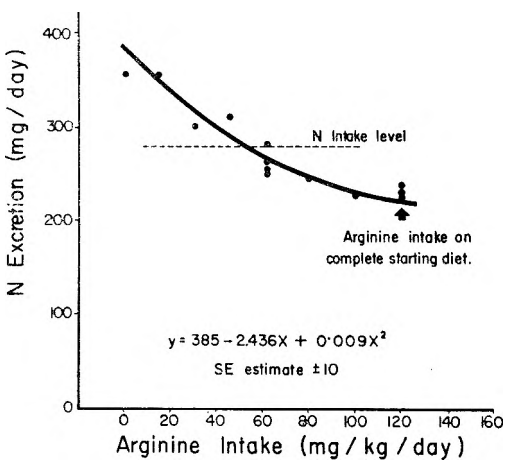

Fig. 2 The effect of arginine intake on nitrogen excretion in adult roosters receiving a free amino acid diet, mean values for 4 birds. Dotted line represents total N intake of 280 mg/kg/day.

DISCUSSION

The requirements observed in the present study demonstrate the importance of considering species and age in amino acid

investigations. The lack of a maintenance requirement for glutamic acid is not surprising because of the ease of synthesis of this amino acid in normal metabolic pathways. In this regard the mature rooster is similar to the adult human (Rose, '57) and rat (Burroughs et al., '40). For optimal growth and in the presence of the *minimal* number of amino acids, the chick (Almquist and Grau, '44), the rat (Rose et al., '48) and the mouse (Maddy and Elvehjem, '49) do require glutamic acid in their diet. Johnson and Fisher ('56) have also shown that this amino acid is required for normal egg production in the hen.

Observations concerning the histidine requirement generally follow those for glutamic acid. Again the requirement for the adult rooster follows that established for the adult human (Rose et al., '51) and the adult rat (Burroughs et al., '40) in that this amino acid is not required. Moore and Wilson ('57), however, concluded that histidine was essential for the maintenance of nitrogen balance in the adult rat. A possible explanation of these discrepancies might be the findings of Nasset and Gatewood ('54) that the low requirement for histidine can be met for short periods of time by the degradation of hemoglobin. The roosters used in the present studies did not show any decrease in hemoglobin concentration during an 8-day experimental period. For growth in the rat and chick as well as for egg production purposes histidine is an established essential amino acid (Rose et al., '48; Scott, '58; Johnson and Fisher, '56).

Although Burroughs et al. ('40) proposed that lysine was not essential for the maintenance of nitrogen balance in the adult rat both the adult human (Rose et al., '55) and the adult rooster (present study) appear to have a definite but small requirement. It is of interest to note that the minimal level necessary to maintain balance in the adult human when calculated on a basis of milligrams per kilogram per day is of the same order of magnitude as determined in this study for the adult rooster.[4]

The arginine requirement of the chicken contrasts sharply with similar requirements of the rat and human. Not only does the growing chick have a large requirement for this amino acid but it remains a most important essential in the diet of the mature rooster as well. Womack and Rose ('47) showed that proline and glutamic acid are convertible to arginine and exert a sparing action on this amino acid in the growing rat. These synthetic pathways appear to be absent or of little consequence in the chick and adult rooster (Fisher et al., '59). It has also been demonstrated that ornithine, an intermediate of the urea cycle, will not spare arginine in the chick (Klose et al., '38). These findings all point towards a unique pathway of arginine metabolism in the chicken.

The disproportionately large arginine requirement of the mature rooster, particularly in relationship to the lysine requirement, may be partly explained by the greater demand for amino acids for keratin synthesis in the replacement of feathers. A similar hypothesis has been proposed as a reason for the difference in amino acid requirements between the adult rat and adult human (Mitchell, '50). In the rooster this explanation is supported by the composition of feathers which contain large amounts of arginine (8.0 gm/16 gm nitrogen), less lysine (1.8 gm/16 gm nitrogen) and considerably less histidine (0.6 gm/16 gm nitrogen), Block and Bolling ('51). The validity of this concept will be further tested by the determination of the requirements for the remaining amino acids, particularly the sulfur-containing amino acids, methionine and cystine.

SUMMARY

The maintenance requirements for L-glutamic acid, L-histidine, L-lysine and L-

[4] Lysine requirement for the adult human calculated as indicated: Rose et al. ('55) in young men, assuming a body wt. of 70 kg, 7.5 to 11.4 mg/kg/day; Jones et al. ('56) in women, assuming a body wt. of 60 kg, 6.7 to 8.3 mg/kg/day; Clark et al. ('57) for men and women, 8.0 to 11.9 mg/kg/day. Also of interest is the similarity in the increased caloric needs of the adult human and chicken receiving free amino acid diets. Rose ('57) states that the replacement of protein by a free amino acid mixture increased the caloric requirement by 10 to 20 Cal./kg, this value is in good agreement with the 20 Cal./kg increase previously observed in the adult rooster (Leveille and Fisher, '58).

arginine have been determined for the adult rooster not previously depleted of his protein reserves using a free amino acid diet. Using a 5-day feeding period nitrogen balance was employed as a criterion of adequacy.

Since the same amount of diet, differing only in quantity of test amino acid (with compensatory nitrogen), was fed, the *requirement* for an amino acid was taken as that level of an amino acid which would maintain the same level of nitrogen excretion as observed on the complete starting amino acid diet. The *minimal maintenance level* was taken as the lowest level of an amino acid which would maintain the animal in nitrogen balance.

L-Glutamic acid and L-histidine were found to be non-essential as judged by either criterion. The requirement for L-lysine was found not to exceed 29 mg/kg/day and no minimal maintenance level could be demonstrated.

The requirement for L-arginine was observed to be 120 mg/kg/day with a minimal maintenance level of 54 mg/kg/day. The relationship of the amino acid requirements for feather synthesis to the maintenance requirement of the rooster have been discussed.

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LITERATURE CITED

Allison, J. B. 1951 Interpretation of N balance data. Federation Proc., *10:* 676.

Almquist, H. J., and C. R. Grau 1944 The amino acid requirements of the chick. J. Nutrition, *28:* 325.

Ariyoshi, S. 1957 Studies on the nitrogen metabolism in the fowl. II. Protein requirement and amino acid balance for maintenance. Bull. Nat. Inst. Agr. Sci., G 12: 93.

Block, R. J., and D. Bolling 1951 The Amino Acid Composition of Proteins and Foods. Charles C Thomas, Springfield, Illinois.

Burroughs, E. W., H. S. Burroughs and H. H. Mitchell 1940 The amino acids required for the complete replacement of endogenous losses in the adult rat. J. Nutrition, *19:* 363.

Clark, H. E., E. T. Mertz, E. H. Kwong, J. M. Howe and D. C. DeLong 1957 Amino acid requirements of men and women. I. Lysine. Ibid., 62: 71.

Fisher, H., and D. Johnson, Jr. 1956 The amino acid requirement of the laying hen. I. The development of a free amino acid diet. Ibid., *60:* 261.

Fisher, H., G. A. Leveille and P. Griminger 1959 The indispensable nature of arginine for maintenance in the rooster and its irreplaceability for chick growth by proline and glutamic acid. Poultry Sci., *38:* 231.

Fritz, J. C., W. A. Hendricks and H. W. Titus 1936 The influence of previous feeding on the N excretion of fasting birds. Am. J. Physiol., *115:* 281.

Johnson, D. Jr., and H. Fisher 1956 The amino acid requirement of the laying hen. II. Classification of the essential amino acids required for egg production. J. Nutrition, 60: 275.

Jones, E. M., C. A. Baumann and M. S. Reynolds 1956 Nitrogen balances of women maintained on various levels of lysine. Ibid., *60:* 549.

Klose, A. A., E. L. Stokstad and H. J. Almquist 1938 The essential nature of arginine in the diet of the chick. J. Biol. Chem., *123:* 691.

Leveille, G. A., and H. Fisher 1958 The amino acid requirements for maintenance in the adult rooster. I. Nitrogen and energy requirements in normal and protein-depleted animals receiving whole egg protein and amino acid diets. J. Nutrition, *66:* 441.

Maddy, K. H., and C. A. Elvehjem 1949 Studies on growth of mice fed rations containing free amino acids. J. Biol. Chem., *177:* 577.

Mitchell, H. H. 1950 Some species differences in amino acid requirements. In: Protein and Amino Acid Requirements of Mammals, edited by A. A. Albanese. Academic Press, New York.

Moore, T. B., and J. E. Wilson 1957 Histidine as an essential nutrient for the adult rat. J. Nutrition, 62: 357.

Nasset, E. S., and V. H. Gatewood 1954 Nitrogen balance and hemoglobin of adult rats fed amino acid diets low in L- and D-histidine. Ibid., *53:* 163.

Rose, W. C. 1957 The amino acid requirements of adult man. Nutrition Abstrs. Revs., 27: 631.

Rose, W. C., A. Borman, M. J. Coon and G. F. Lambert 1955 The amino acid requirements of man. X. The lysine requirement. J. Biol. Chem., *214:* 579.

Rose, W. C., J. W. Haines and D. T. Warner 1951 The amino acid requirements of man. III. The role of isoleucine: additional evidence concerning histidine. Ibid., *193:* 605.

——— 1954 The amino acid requirements of man. V. The role of lysine, arginine and tryptophan. Ibid., *206:* 421.

Rose, W. C., M. J. Osterling and M. Womack 1948 Comparative growth on diets containing 10 and 19 amino acids, with further observations upon the role of glutamic and aspartic acids. Ibid., *176:* 753.

Scott, H. M. 1958 Observations on the amino acid requirements of chicks fed crystalline amino acids. Proc. Cornell Nutr. Conf., p. 106.

Womack, M., and W. C. Rose 1947 The role of proline, hydroxyproline and glutamic acid in growth. J. Biol. Chem., *171:* 37.

Methionine, Iodocasein and Oxygen Consumption of Chicks[1]

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The metabolic roles of amino acids are currently under intensive and extensive study. In some instances it appears that the primary view of amino acids as substrates for protein synthesis must be enlarged to contemplate other functions, even in young, rapidly growing animals. Some amino acids, while indeed serving as substance for tissue formation, have equally interesting roles other than this. Thus they fit the vitagen concept of Rosenberg ('45). One such amino acid appears to be methionine, in view of its role in transmethylation and in view of the nutritional findings of the present paper.

For several years in the author's laboratory an effort has been in progress to elucidate the role of vitamin B_{12} in amino acid metabolism, as affected by stresses applied to experimental chicks. One such stress factor is the thyroid hormone or dietary sources of it such as iodinated casein.[2] A great deal of work has established that iodocasein may be regarded as a convenient dietary source of this hormonal activity. Further it will be recalled that iodocasein increases the chick's requirement for vitamin B_{12} (Robblee et al., '48); also that liver and other sources of "animal protein factor," or vitamin B_{12} itself alleviate the thyrotoxicosis induced by iodinated proteins (Nichol et al., '49). All of this pointed to the possibility of some metabolic balance between this vitamin and the thyroid hormone.

While the functioning of the thyroid hormone is not entirely clear, it is beyond doubt that it increases the metabolic rate and oxygen consumption of animals. It also is well established that beyond small and very critical levels the thyroid hormone is a metabolic uncoupler, dissociating anabolic tissue-building reactions from oxidative reactions which are the source of the necessary energy (Maley and Lardy, '55). The animal is made to "spin the wheels" so to speak; oxidizing foodstuffs, radiating heat, but not gaining weight as well as controls for the amount of feed consumed. And yet this picture sometimes can be reversed. If the level of hormone is low enough and if the state of nutriture is adequate, additional gains can be realized by the use of dietary iodinated proteins (Arscott and Combs, '55).

Bird, Rubin and Groschke ('47) noted that methionine had a beneficial effect in diets rich in soybean protein. Rubin and Bird ('47) suggested that the animal protein factor (vitamin B_{12}) might conceivably exert its effects by facilitating liberation of methionine from soybean protein. While some effects of vitamin B_{12} in one-carbon metabolism (and other aspects) have been established subsequently (Broquist, '58), the full explanation of its nutritional value probably eludes us still.

In some preliminary experiments in this laboratory aimed at clarifying the relationships between iodocasein, vitamin B_{12} and methionine, vitamin B_{12} reversed some of the effects of iodocasein, notably in preventing development of oversize livers. But more interestingly, methionine reversed the effect of iodocasein on oxygen uptake of the intact chicks, while vitamin B_{12} did not. These findings have been confirmed in two repeat experiments with a more precisely defined diet and more refined technique for measurement of oxygen consumption. The present paper is a presentation of these two experiments.

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[2] Protamone.

PROCEDURE

The animals used were Single Comb White Leghorn cockerels. They were purchased from a west coast hatchery for experiment 33, from a local hatchery for experiment 34. Experimental groups were selected after a two-day stabilizing period on corn meal, by a systematic weight-equalization procedure, and placed in individually heated, wire-mesh-bottom, all metal pens in batteries housed in an air-conditioned, constant temperature animal room. There were 20 chicks per group in experiment 33; 18 per group in experiment 34. They were fed and watered ad libitum. The basal diet consisted, in per cent, of yellow corn meal, 66.0; soybean meal (50% protein), 13.5; dehydrated alfalfa meal (17% protein), 5.88; dried brewers' yeast, 5.00; gelatin, 4.00; steamed bone meal, 1.70; limestone, 1.00, DL-phenylalanine, 0.214; DL-tryptophan, 0.041; iodized salt, 0.500; potassium chloride, 0.200; magnesium sulfate, 0.242; and vitamins and trace minerals in milligrams per kilogram of diet as follows: $MnSO_4$, 50; $FeSO_4$, 20; $CuSO_4$, 2.0; $ZnCl_2$, 0.2; $CoCl_2$, 0.2; pyridoxine·HCl, 2.50; folic acid, 0.50; biotin, 0.10. Calculations based on reported compositions of the feedstuffs used indicated that this diet contained 19.7% protein, provided all non-added vitamins and minerals in abundance, and all essential amino acids except methionine, histidine and isoleucine at levels within 98 to 118% of the National Research Council ('54) requirement levels for a 20% protein diet for chicks. Histidine was present at three times the requirement level; isoleucine at 135% of the requirement; and methionine, after correction for the simultaneous cystine deficiency, at only 36% of the requirement level.

The dietary variables in a 2^3 factorial design were vitamin B_{12} at 25 μg per kilogram of diet, iodocasein at 0.05%, and DL-methionine at 0.362% of the diet. This was the calculated amount to bring total methionine to the requirement level in supplemented groups, discounting the probable effect of the D-isomer. To the extent the D-isomer is active, its presence gave a higher supplementation than that stated.

Experimental criteria were as follows: blood, liver and excreta contents of free methionine, histidine and isoleucine; oxygen uptake of intact birds; body weight gain; feed utilization efficiency; weight of excreta voided; 70% alcohol-insoluble nitrogen and uric acid contents of excreta; and liver as percentage of body weight.

Oxygen uptake measurements were made by the method of Charkey and Thornton ('59). Excreta were handled and analyses made thereon as follows: the droppings were collected near the end of the feeding trial during the same 24-hour collection period in all pens, in stainless steel cafeteria trays levelled under the chicks in the dropping trays and kept full of alcohol by replacement of evaporative losses by absolute ethanol. It is assumed that this "pickling" procedure by the neutral solvent and protein precipitant stopped microbial action as soon as the droppings were voided, and also prevented any extensive hydrolysis of fecal or urinary constituents. Each collection was transferred by washing with a large volume of 70% alcohol into a liter beaker, in which it was thoroughly macerated by a high speed overhead stirrer fitted with a cutting blade. Filtration and washing were accomplished on a large Büchner funnel with suction. The residues were allowed to dry thoroughly in room air, and weighed. Determinations of total solids indicated that all had essentially the same moisture content. Strongly alkaline extracts (NaOH, pH 12–13) were made on aliquots, and used for measurement of uric acid by the method of Benedict and Franke ('22). Other aliquots of the finely ground residues were used for nitrogen determination by the macro-Kjeldahl method. The filtrates were concentrated to dryness without overheating, taken up in distilled water, adjusted to pH 5.0, filtered, and used for microbiological determination of amino acids and for total NPN determinations.

RESULTS AND DISCUSSION

In table 1 are shown averages of O_2 uptake data for both experiments. The "t" test was applied to the pooled sub-means from which these averages were calculated, to test for significance of mean differences

TABLE 1

Oxygen consumption (O_2 uptake) of intact chicks

Pen	treatment[1]	Experiment APF-33 (Feb. 20–Mar. 20) (age in days)			Experiment APF-34 (June 12–July 11) (age in days)		
		10	14	21	8	15	22
		(ml of dry oxygen per kg chicks per second)[2]					
1	Basal	0.557	0.590	0.491	0.446	0.510	0.434
2	Vitamin B_{12}	0.638	0.629	0.492	0.493	0.567	0.452
3	DL-Methionine	0.648	0.585	0.459	0.486	0.429	0.405
4	Vitamin B_{12} plus methionine	0.626	0.588	0.451	0.505	0.447	0.408
5	Iodocasein	0.592	0.660	0.580	0.490	0.612	0.568
6	Iodocasein plus vitamin B_{12}	0.628	0.645	0.563	0.515	0.581	0.602
7	Iodocasein plus methionine	0.665	0.622	0.478	0.481	0.512	0.532
8	Iodocasein plus vitamin B_{12} plus methionine	0.710	0.631	0.493	0.570	0.571	0.515

[1] Note on levels: Vitamin B_{12} at 25 μg per kilogram of diet; iodocasein (Protamone) at 0.05% of diet; DL-Methionine at 0.362% of diet (to meet the NRC requirement, considering only the L-isomer).

[2] This simpler unit for O_2 uptake values is used throughout this paper, inasmuch as recalculation using body weight to the ¾ power instead of body weight did not materially affect results obtained.

found. In every case except one which is indicated, the overall effect of a single supplement was studied. Specifically, for example, to test the effect of iodocasein on O_2 uptake, a mean of data from all groups supplemented with iodocasein was compared to a similar mean from all groups not supplemented with iodocasein. Use of the "t" test in this manner disclosed the following: at 10 days of age (8 days in exp. 34) all supplements produced increases in O_2 uptake in both experiments. These were of the order of 5 to 10% increase due to supplementation. Only one of these, that due to vitamin B_{12} in experiment 34, proved highly significant ($P > 99\%$). This early effect of vitamin B_{12} is interesting in view of the fact that from two weeks of age on, the vitamin failed to produce any significant effect on O_2 uptake in any section of either experiment.

At two weeks and thereafter a consistent pattern was in evidence. Iodocasein increased O_2 uptake, and methionine decreased it, until termination at 4 weeks in both experiments.

In experiment 34 the iodocasein produced larger increases than in experiment 33; so that at three and 4 weeks of age (exp. 34), while methionine still decreased O_2 uptake, the differences were no longer significant in overall comparisons. At this stage the "t" values for iodocasein effects were very high (3 to 4 times that required for significance at the 99% probability level). Accordingly the methionine effects were tested separately in the absence and presence of iodocasein, and proved to be highly significant under both conditions at three weeks. At 4 weeks, however, the methionine effect was lost in the presence of iodocasein, and of doubtful significance in its absence. Thus it is possible that the methionine effect in reducing O_2 uptake of chicks appears only between two and 4 weeks of age in Leghorn chicks. It could nevertheless be a matter of importance to their future development. The iodocasein effect would appear to be of longer duration, but possibly could be overcome by higher levels of added methionine. Further studies are contemplated to elucidate these points.

In table 2 the O_2 uptakes at three weeks are compared to other criteria of reponse. In both experiments body weight gain and

TABLE 2

Comparison of O_2 uptake to other criteria of response

Pen treatment	21st day[1] O_2 uptake	14 day total gain	28 day total gain	14 day FUE[2]	28 day FUE[2]	28th day liver weight	14 day excreta weight	28 day excreta weight	27th day alc.-insol. excreta N	27th day alc.-insol. excreta uric acid N
	ml/kg/sec	*kg*	*kg*	*%*	*%*	*% body wt.*	*kg*	*kg*	*%*	*%*
Experiment 33										
1 Basal	0.491	1.66	4.17	43.7	40.5	2.65	1.20	3.525	6.598	3.72
2 Vitamin B_{12}	0.492	1.79	4.00	46.8	43.0	2.58	1.14	3.23	6.339	3.71
3 DL-Methionine	0.459	1.89	3.72	48.9	44.6	2.31	1.14	2.76	5.996	3.53
4 Vitamin B_{12} + methionine	0.451	1.92	3.25	48.8	42.0	2.35	1.16	2.595	6.174	3.51
5 Iodocasein	0.580	1.28	3.38	40.4	38.9	3.23	0.943	3.22	6.530	3.80
6 Iodocasein + vitamin B_{12}	0.563	1.52	3.62	43.8	40.3	2.78	1.07	3.39	6.320	3.47
7 Iodocasein + methionine	0.478	1.77	4.12	48.5	44.4	2.69	1.12	3.38	5.895	3.065
8 Iodocasein + vitamin B_{12} + methionine	0.493	1.78	3.76	48.1	41.9	2.66	1.13	3.13		
Experiment 34[3]										
1 Basal	0.434	1.37	3.87	38.0	37.6	2.67	1.31	3.40	5.442	3.38
2 Vitamin B_{12}	0.452	1.39	3.99	39.25	38.2	2.59	1.08	3.93	5.591	3.22
3 DL-Methionine	0.405	1.485	4.39	40.9	41.6	2.48	1.145	4.01	5.270	2.76
4 Vitamin B_{12} + methionine	0.408	1.52	4.77	44.3	43.5	2.51	1.06	4.05	5.194	2.49
5 Iodocasein	0.568	0.945	3.09	33.6	35.9	2.95	0.858	3.39	5.549	2.89
6 Iodocasein + vitamin B_{12}	0.602	1.12	3.64	35.8	38.0	2.99	0.848	4.02	5.485	2.72
7 Iodocasein + methionine	0.532	1.37	3.56	37.6	37.6	2.72	1.02	3.92	5.295	2.52
8 Iodocasein + vitamin B_{12} + methionine	0.515	1.34	3.92	40.2	37.7	2.81	1.045	4.44	5.428	2.78

[1] In experiment 34, 22nd day.

[2] Feed utilization efficiency. This equals $\frac{\text{total gain in body weight}}{\text{total weight of feed consumed}} \times 100$.

[3] Experiment 34 data, actually based on 18 chicks, multiplied by 20/18 for direct comparison to experiment 33 data (in gain and excreta weight columns).

TABLE 3

Correlation coefficients relating O_2 uptake to other criteria of response

Comparison of O_2 uptake with:	Values of r[1]	
	Exp. 33	Exp. 34
Reciprocal of 14-day total gain	0.944	0.865
Reciprocal of 28-day total gain	+[2]	0.775
Reciprocal of 14-day feed utilization efficiency	0.922	0.874
Reciprocal of 28-day feed utilization efficiency	+[2]	0.835
28th day liver as per cent of body weight	0.898	0.964

[1] r required for significance: 0.666 for 80% probability; 0.798 for 99% probability.
[2] Not calculated. See text.

feed utilization efficiency were inversely related to the O_2 uptakes. That this was not true of body weights at 4 weeks in experiment 33 is not considered to invalidate the general relationship stated, in view of the following: the lack of expected extra growth from methionine at 4 weeks was clearly accompanied by a drop in feed consumption and in excreta voided for the same period. It seems clear that the chicks were off feed for some unknown reason during the third week in groups 3, 4 and 8 of experiment 33. It is fortunate that both feed and droppings were weighed weekly, so that the seemingly anomalous results in this part of experiment 33 have an explanation.

Liver weight as percentage of body weight was directly related to O_2 uptake. Correlations for this and the foregoing relationships proved to be highly significant, as appears in table 3.

In brief, iodocasein caused decreases in gain, associated with enlarged livers and increased O_2 uptake. Methionine produced increases in gain, associated with decreases in liver size and decreased O_2 uptake. This occurred both in the presence and absence of iodocasein. Thus the effects of administered iodocasein resembled in some respects those of methionine deficiency; and supplementary DL-methionine was corrective in both cases.

Examination of the data on free amino acid levels disclosed no consistent relationships to dietary supplements except that methionine supplementation was reflected in blood and excreta levels of methionine. One purpose of these experiments was to learn if vitamin B_{12} would enhance conversion of an amino acid in excess (histidine or isoleucine) to one in deficiency (methionine). The data on free amino acids provide no indication that it did so in these cases. Nor did iodocasein appear to affect amino acid interrelationships in any way observable from their levels in blood, liver and excreta.

Weights of excreta voided were roughly parallel to feed consumed and growth produced. No specific relationship to any dietary supplementation is in evidence.

Total nitrogen and uric acid values in alcohol-extracted excreta tended to be inversely related to methionine supplementation and to growth obtained, except for the 28-day gains in experiment 33. The lack of growth response to methionine here in groups 3, 4 and 8 resulting from reduced feed intake has already been pointed out. Despite this, the relationship stated seems generally well supported. The percentage reduction in total nitrogen appears to be attributable almost entirely to the reduction in uric acid nitrogen. Since uric acid is a principal end-product of nitrogen metabolism in avian species, its greater excretion in methionine deficiency probably resulted from diversion of nitrogen from tissue protein deposition to excretory channels. This might be expected in any of various amino acid imbalances, and is not considered to be of any special significance in relation to methionine or to iodocasein. The data may, however, indicate a specific relationship to vitamin B_{12}, which vitamin is known to play a role in purine biosynthesis, since uric acid excretion appeared to be slightly reduced by vitamin B_{12} supplementation.[3]

Here again there is the suggestion arrived at in other ways in earlier experiments (Charkey et al., '50, '53, '54) that

[3] In this connection it should be recalled that the method used for uric acid measurement is not considered highly specific for uric acid (Hawk et al., '54). The reported values perhaps should be regarded as measures of total alcohol-insoluble purines in samples of this origin. Non-protein nitrogen measurements in the alcohol extracts (the same as used for excreta amino acids) from these samples showed no trend.

vitamin B_{12} has a role in metabolism of amino acids such as to make their utilization more efficient under stress. In the present work the stress was a dietary amino acid imbalance, leading to nitrogen wastage through uric acid excretion.

SUMMARY

A factorial design, using vitamin B_{12}, DL-methionine and iodocasein with a vitamin B_{12} deficient, methionine-deficient basal diet, has been used to compare the effects of these supplements on oxygen consumption and amino acid utilization in chicks. The basal diet was balanced with respect to essential amino acids except for methionine, grossly deficient, and histidine and isoleucine, which were in excess.

Iodocasein decreased growth and feed efficiency, increased oxygen consumption of the intact animals, and also the weight of their livers compared to body weight. Methionine reversed all of these effects of iodocasein. Further, methionine in the absence of iodocasein had effects on the foregoing criteria opposite from those of iodocasein.

Since thyroid hormone is a metabolic uncoupler it is suggested that methionine functions in metabolism as a coupling agent or by enhancing biosynthesis of such an agent, or by interfering otherwise with the functioning of the thyroid hormone as an uncoupler. Thus methionine may be a factor in linking anabolic reactions to oxidative reactions, thereby making available the energy provided by the latter for more effective animal function. And thus there is here a possible basic explanation of the results of its deficiency.

Methionine, and to some extent vitamin B_{12}, reduced excretion of uric acid from that found in controls. In the case of methionine this was concomitant with an obvious correction of methionine deficiency. Hence the reduction of uric acid excretion by vitamin B_{12} in the absence of added methionine suggests that the vitamin also corrected the amino acid imbalance to some extent. This is consistent with the findings that vitamin B_{12}, like methionine but to a lesser degree, also improved growth and feed utilization efficiency, and reduced liver size.

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LITERATURE CITED

Arscott, G. H., and G. F. Combs 1955 Unidentified growth factors required by chicks and poults. Poultry Sci., *34:* 843.

Benedict, S. R., and E. Franke 1922 A method for the direct determination of uric acid in urine. J. Biol. Chem., *52:* 387.

Bird, H. R., M. Rubin and A. C. Groschke 1947 An inborn characteristic determining the response of chickens to a diet free of animal protein. J. Nutrition, *33:* 319.

Broquist, H. P. 1958 Water soluble vitamin. I. Folic acid, B_{12} group and choline. Ann. Rev. Biochem., *27:* 285.

Charkey, L. W., A. K. Kano and J. A. Anderson 1954 Effects of fasting on free amino acid levels in chick blood as modified by vitamin B_{12}. J. Biol. Chem., *210:* 627.

Charkey, L. W., W. K. Manning, A. K. Kano, F. X. Gassner, M. L. Hopwood and I. L. Madsen 1953 A further study of vitamin B_{12} in relation to amino acid metabolism in the chick. Poultry Sci., *32:* 630.

Charkey, L. W., and P. A. Thornton 1959 A simple method for estimating oxygen consumption of small animals. Ibid., *38:* 899.

Charkey, L. W., H. S. Wilgus, A. R. Patton and F. X. Gassner 1950 Vitamin B_{12} in amino acid metabolism. Proc. Soc. Exp. Biol. Med., *73:* 21.

Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry (p. 905) Blakiston Company., Inc., New York.

Maley, G. F., and H. A. Lardy 1955 Efficiency of phosphorylation in selected oxidations by mitochondria from normal and thyrotoxic rat livers. J. Biol. Chem., *215:* 377.

National Research Council Publication 301 1954 Nutrient Requirements for Poultry.

Nichol, C. A., L. S. Dietrich, W. W. Cravens and C. A. Elvehjem 1949 Activity of vitamin B_{12} in the growth of chicks. Proc. Soc. Exp. Biol. Med., *70:* 40.

Robblee, A. R., C. A. Nichol, W. W. Cravens, C. A. Elvehjem and J. G. Halpin 1948 Relation between induced hyperthyroidism and an unidentified chick growth factor. Ibid., *67:* 400.

Rosenberg, H. R. 1945 Chemistry and Physiology of the Vitamins. Interscience Publishers, Inc., New York.

Rubin, M., and H. R. Bird 1947 A chick growth factor in cow manure. V. Relation to quantity and quality of soybean oil meal in the diet. J. Nutrition, *34:* 233.

Serum Protein Changes in Vitamin E-Deficient Chicks

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The first symptom which appears in chicks fed certain diets deficient in vitamin E is exudative diathesis, a syndrome characterized by a subcutaneous, hemorrhagic edema over the breast. In addition to vitamin E, trace amounts of selenium also prevent this condition (Patterson et al., '57; Schwarz et al., '57). Although Dam and Glavind ('40) had originally concluded that capillary damage was the initial lesion involved, Goldstein and Scott ('56) have recently observed alterations in the serum proteins of vitamin E-deficient chicks, raising the question whether osmotic changes in the vascular system may not contribute to the edema. Subsequent studies with turkeys (Creech et al., '57) and with chicks (Dam et al., '57) have confirmed that the serum proteins are abnormal in birds with this syndrome. Creech et al. ('58) have shown further that changes in the serum proteins precede the onset of exudative diathesis.

Because the rate of depletion of vitamin E, and hence the rate of appearance of symptoms, varies considerably in any group of chicks, it was felt that a more definitive picture of the sequence of changes in exudative diathesis could be obtained by following the progress of the deficiency in individual birds. In the present study, it is shown that although changes in serum proteins are seen several days before symptoms appear, these changes do not appear to be the precipitating factor in the formation of exudates. More marked changes in the proteins are found during the period of spontaneous recovery when the edema disappears. Normal protein patterns are often reestablished without vitamin E treatment.

EXPERIMENTAL

Blood (1.0 ml) was taken by heart puncture and the individual serums were separated and stored at 4°C. Total serum protein concentration was determined colorimetrically by the biuret procedure of Gornall et al. ('49). Changes in the various protein fractions were determined in 10 μl samples of serum by paper electrophoresis with the Beckman Spinco Model R apparatus. Barbiturate buffer of ionic strength 0.075 and pH 8.6, a current of 8 ma, and a running time of 16 hours were used. The electrophoretic patterns of the stained proteins were traced with the Spinco Analytical Scanner and Integrator. In calculating albumin: globulin (A/G) ratios, all of the area behind the albumin peak was considered to be globulin. Since the patterns obtained with chicken serum show several more peaks than those obtained with human or rat serum under these electrophoretic conditions, and since the minor fractions usually were not clearly separated, it was not considered feasible to attempt to calculate concentrations of individual fractions. To eliminate the effects of possible variations in electrophoretic conditions, the serum samples from a single chick, which were to be compared with each other, were stored in the refrigerator and eventually run simultaneously.

New Hampshire chicks one day old were fed a purified, vitamin E-free diet (C47C) containing in per cent: soybean protein,[1] 30; torula yeast, 15; stripped lard,[2] 4; salt mixture (Schwarz et al., '57), 6; vitamin

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[1] Drackett Assay Protein C–1.

[2] Distillation Products Industries, Rochester, New York.

mix,[3] 0.2; L-cystine, 0.3; DL-methionine, 0.2; glucose, 44.3. On this diet, exudative diathesis appeared in 80% of the birds in 14 to 25 days, at which time weights were from 125 to 300 gm. Several complete diets which supported normal growth and development were used: diet S, a commercial starting ration; diet C50, a purified diet containing 20% purified casein, 8% gelatin, 4% corn oil, 6% salt mixture, 61.5% glucose, 0.3% DL-methionine, and all vitamins; diet C47C above with 100 mg of α-tocopheryl acetate/kg; diet C47C with the torula yeast omitted and with 0.5 ppm of selenium added. Chicks were examined twice daily (9:00 A.M. and 4:00 P.M.) for the appearance of exudates. Selenium was added to diets as sodium selenite.

RESULTS

Electrophoretic patterns of normal chick serum

In order to establish the normal serum protein pattern in chicks under our experimental conditions, analyses were made of serums from 14- to 21-day-old chicks which had received 4 different complete diets. Analyses of serums from 9 chicks, two or three on each diet, gave an average total protein content of 3.52 ± 0.7% and an average A/G ratio of 0.425 ± 0.16. The electrophoretic patterns of the serums with the highest and lowest A/G ratios are shown in figure 1.

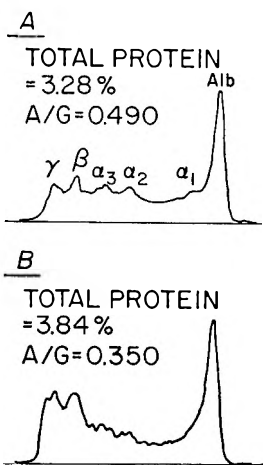

Fig. 1 Normal serum protein patterns. A, 14-day-old chick fed a commercial chick ration. B, 27-day-old chick fed a synthetic vitamin E-free diet (C47C) containing 0.5 ppm of selenium.

In almost all serums examined, whether from normal or deficient chicks, 6 to 8 peaks were discernible. The designation of individual protein areas considered suitable for this study is shown in figure 1A. Similar patterns have been reported by Vanstone et al. ('55). These workers also showed that from the 14th to 35th day of life the total serum proteins increased only slightly with no change in the A/G ratio. A similar relatively constant composition of the serum proteins in normal chicks of this age period was also observed in the present study.

Electrophoretic changes with the onset of exudates

In order to observe the sequence of changes in the serum proteins during the development of exudative diathesis, 24 chicks fed the vitamin E-deficient diet C47C were bled beginning on the 10th day. Symptoms usually began to appear after the 12th day on this diet. The chicks were bled twice prior to the appearance of symptoms, some on the 10th and 11th days while others were bled on the 10th and 12th days. If the chicks did not develop exudates within two days after the second bleeding, they were discarded. It was felt that further bleeding would give rise to blood changes due to excessive loss of volume. There was no indication that the one or two bleedings either retarded or accelerated the development of exudates.

Serial samples of serum were thus obtained from 6 chicks. Analyses revealed that two to three days prior to the appearance of symptoms, total serum proteins and the A/G ratios were in the normal range (table 1 and fig. 2). When the serums from the same chicks were analyzed as soon as exudates were noted, the A/G ratios had fallen considerably, while the decrease in total proteins was only slight for 4 chicks, with a significant decrease for one and a slight increase for another. In two chicks whose blood was sampled 24 and 48 hours before symptoms appeared, there was an indication that the decrease in A/G ratio began more than 24 hours before the edema occurred.

[3] Containing adequate amounts of all vitamins but A and E. Vitamin A was administered in aqueous solution.

TABLE 1

Changes in the total serum proteins and in the albumin: globulin (A/G) ratios one to three days prior to the appearance of exudates in vitamin E-deficient chicks

Chick no.	Time before exudate appeared					
	48–72 hours		16–24 hours		0[1]	
	Total protein	A/G	Total protein	A/G	Total protein	A/G
	%		%		%	
1	3.44	0.478	—	—	3.94	0.346
2	3.08	0.531	—	—	2.92	0.327
3	4.32	0.367	3.82	0.252	2.96	0.202
4	3.22	0.470	3.38	0.354	3.24	0.368
5	—	—	3.15	0.405	2.92	0.343
6	—	—	2.88	0.458	2.72	0.258

[1] Sample taken when exudate was first noted.

Examination of the individual electrophoretic patterns showed that, in 5 of the 6 chicks one or two days prior to the appearance of symptoms, the overall protein picture was not greatly altered from the normal. The only obvious change in three of the serums was an increase in the α_2- and α_3-globulin areas. This can be seen in figure 2, E and F.

Serum changes after spontaneous recovery from exudative diathesis

One of the unique aspects of this deficiency syndrome is the spontaneous recovery which frequently occurs when the diet is not strongly pro-oxygenic. That is, with no change in dietary or other conditions, the edematous fluid is completely resorbed and the bird appears normal in all outward respects. This may occur rapidly within two or three days, and the exudate may or may not recur. Twelve chicks which had recovered in this manner were bled two to 8 days after the appearance of exudates. In three of these chicks the normal pattern had been re-established. In the other 9 birds, however, it was apparent that marked changes occurred during the recovery phase. Two patterns which repre-

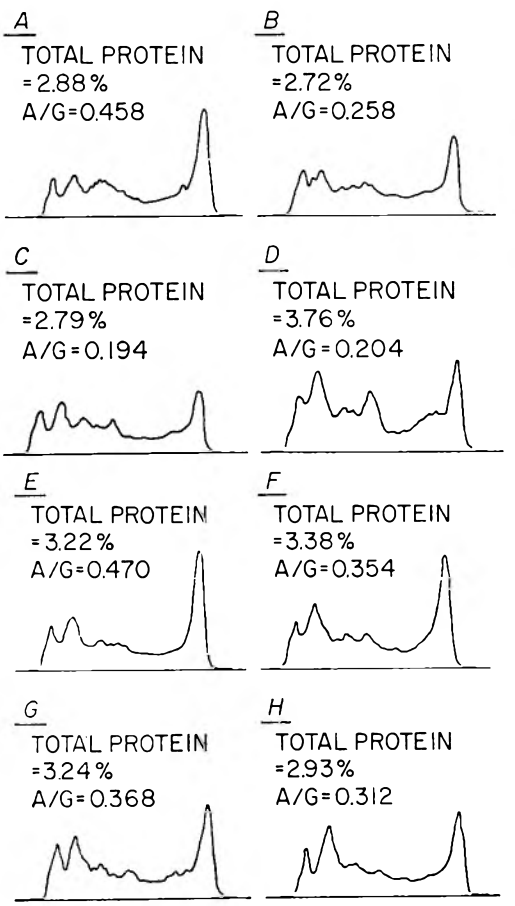

Fig. 2 Sequence of changes in serum protein patterns before, during, and after the appearance of exudates. Chick no. 2806: (A) serum 17 hours before symptoms when chick was still normal; (B) serum taken when a severe exudate was first noted; (C) serum taken 24 hours after sample B, exudate partially resorbed; (D) serum taken 48 hours after sample B, exudate almost completely resorbed. Chick no. 2809: (E) serum 48 hours before the appearance of exudate; (F) serum 16 hours before symptoms, chick still normal; (G) serum taken when chick had mild exudate; (H) serum taken 24 hours after sample G, chick had moderate exudate.

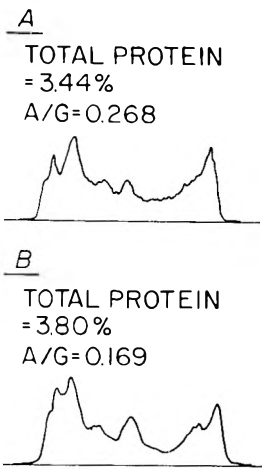

Fig. 3 Serum protein patterns from two chicks which had spontaneously recovered from exudative diathesis. The chicks appeared normal in all respects. A, 6 days after the exudate; B, 5 days after the exudate.

sent the alterations most frequently observed are shown in figure 3. It can be seen that there was a marked increase in the α_2-globulin peak, as well as increases in the α_1-, β-, and γ-globulins. Albumin decreased considerably. Similar changes can be seen in comparing the patterns in figure 2, C and D. Although there was considerable variability, in 8 of the post-exudate serums the most marked change was the increased α_2-globulin. Not all chicks showed a decrease in albumin, however. During the recovery phase, the total protein concentrations were usually elevated, while the A/G ratios varied from normal to very low levels (4 were below 0.20). An occasional chick after recovering would become moribund, cease to eat, and die within a week. In two chicks in this condition the A/G ratios were 0.165 and 0.155. The most abnormal protein pattern found in this entire study was in the group of completely recovered, and apparently normal, chicks: A chick which had had a severe exudate 4 days previously was completely recovered and had a total serum protein of 4.15% and an A/G ratio of 0.072. When chicks which had recovered spontaneously were continued on the deficient diets for longer periods, three to 4 weeks, the serum patterns then gradually returned to normal. Administration of vitamin E or selenium usually restored the normal pattern in about one week.

DISCUSSION

The lowered A/G ratios in chicks with exudates confirms the observations of Goldstein and Scott ('56), Dam et al., ('57), and Creech et al. ('58). The present study, however, by following the changes in individual chicks, provides a clearer picture of the development of the protein abnormalities. It is apparent that, concomitant with the appearance of exudates in vitamin E-deficient chicks, changes occurred in the composition of the serum proteins. The most consistent alteration was a decrease in the A/G ratio during the two or three days prior to the onset of symptoms. A decrease in total proteins was evident in some chicks while in others there was no change. In attempting to determine the relationship of the changes in serum proteins to the formation of exudates, it is necessary to consider the status of the proteins throughout the various stages of the condition. Inasmuch as the A/G ratio during the recovery phase, when the chicks appeared normal, was often lower than when exudates were present or were forming, it is evident that the decreased albumin concentration alone cannot account for the edema. Similarly, it would not appear that a decrease in total serum proteins would be involved, for some chicks with exudates had higher serum protein levels than chicks without symptoms. These observations would rule out osmotic changes as being of primary importance in the development of exudative diathesis. The original hypothesis of Dam and Glavind ('40) that capillary damage is the initial lesion appears to remain as the most logical explanation.

The abnormal serum protein patterns, characterized by a low A/G ratio and high total protein, observed in deficient chicks which recovered spontaneously, are similar to patterns found by Goldstein and Scott ('56). Because of the increase in total protein over the normal level, it is obvious that the low A/G ratio is a result not only of a smaller amount of albumin, but also an increased amount of globulin. It would appear that, during recovery, globulin syn-

thesis proceeds faster than does albumin synthesis.

The condition of exudative diathesis is caused by dietary conditions which lead to the formation of peroxides in the tissues. The most important dietary factors in this respect are unsaturated fat or certain mineral combinations which promote peroxidation of the body lipids (Bieri et al., '58). Either vitamin E or selenium can prevent this. Although the antioxidant nature of α-tocopherol is well known, no similar function for selenium has been described. If the damage to capillaries is caused by products of autoxidation of fatty acids as Dam ('57) has postulated, then it appears necessary to propose a role for selenium in the sequence of reactions which constitutes autoxidation. It seems evident that the mechanism of selenium action, however, must be other than that of an antioxidant in the usual sense.

The re-establishment of a normal serum protein pattern in vitamin E- and selenium-deficient chicks would appear to rule out a direct function for these metabolites in protein synthesis. Further support for this view, in the case of vitamin E, is the observation that chicks depleted of tocopherol for long periods of time (as long as 10 months) continue to grow and function normally (Bieri and Briggs, '59). It has also been shown that in such chicks the activity of certain respiratory enzymes is similar to that of control chicks (Pollard and Bieri, '59). The possibility that traces of the vitamin, or of some product derived from it, may still be present in the tissues after this period seems remote.

SUMMARY

Concomitant with the onset of exudates in chicks fed a diet deficient in vitamin E and selenium, changes occurred in the electrophoretic patterns of the serum proteins. The decrease in albumin: globulin ratio did not appear to be of sufficient magnitude to account for the edema. Total serum proteins declined only slightly, if at all. The most marked alterations in protein patterns developed after the chicks had spontaneously recovered, when increases in α_2-, α_3-, β-, and γ-globulins occurred. The serum protein patterns in deficient chicks were sometimes restored to normal without the administration of vitamin E or selenium.

LITERATURE CITED

Bieri, J. G., and G. M. Briggs 1959 Nonessentiality of vitamin E for normal growth and development of the chick. Federation Proc., *18:* 2033.

Bieri, J. G., G. M. Briggs and C. J. Pollard 1958 Acceleration of vitamin E deficiency by Torula yeast. II. Effect of Torula yeast ash and lipid. Proc. Soc. Exp. Biol. Med., *99:* 262.

Creech, B. G., G. L. Feldman, T. M. Ferguson, B. L. Reid and J. R. Couch 1957 Exudative diathesis and vitamin E deficiency in turkey poults. J. Nutrition, 62: 83.

Creech, B. G., M. M. Rahman, B. L. Reid and J. R. Couch 1958 Exudative diathesis in chicks. Ibid., *64:* 55.

Dam, H. 1957 Influence of antioxidants and redox substances on signs of vitamin E deficiency. Pharmacol. Revs., *9:* 1.

Dam, H., and J. Glavind 1940 Vitamin E and kapillarpermeabilität. Naturwissenschaften, 28: 207.

Dam, H., S. Hartmann, J. E. Jacobsen and E. Sondergaard 1957 The albumin/globulin ratio in plasma and exudate of chicks suffering from exudative diathesis. Acta Physiol. Scand., *41:* 149.

Goldstein, J., and M. L. Scott 1956 An electrophoretic study of exudative diathesis in chicks. J. Nutrition, 60: 349.

Gornall, A. G., C. J. Bardawill and M. M. David 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., *177:* 751.

Patterson, E. L., R. Milstrey and E. L. R. Stokstad 1957 Effect of selenium in preventing exudative diathesis in chicks. Proc. Soc. Exp. Biol. Med., *95:* 617.

Pollard, C. J., and J. G. Bieri 1959 Studies of the biological function of vitamin E. I. Tocopherol and reduced diphosphopyridine nucleotide-cytochrome c reductase. Biochim. Biophys. Acta, *34:* 420.

Schwarz, K., J. G. Bieri, G. M. Briggs and M. L. Scott 1957 Prevention of exudative diathesis in chicks by Factor 3 and selenium. Proc. Soc. Exp. Biol. Med., *95:* 621.

Vanstone, W. E., W. A. Maw and R. H. Common 1955 Levels and partition of the fowl's serum proteins in relation to age and egg production. Can. J. Biochem. Physiol., *33:* 891.

The Availability to Chicks of Zinc in Various Compounds and Ores[1]

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The production of zinc deficiency in the chick and the description of the symptoms that result from this deficiency by O'Dell and Savage ('57) have stimulated research work on this element in poultry nutrition. A number of compounds have been used by various workers as a source of zinc in poultry rations. However, no detailed study has been carried out to determine the availability of zinc in various compounds and ores.

The studies reported in this paper were conducted to determine the relative availability to the young growing chicken of zinc in various chemical compounds and mineral ores.

EXPERIMENTAL

Day-old Plymouth Rock cockerels were obtained from a commercial source and individually wing-banded. These chicks were weighed at weekly intervals during the course of the two-week experimental period. Environmental zinc was reduced to a minimum by housing the birds in wooden cages covered with plastic on pine shaving litter. Heat was supplied with infra-red lamps. The chicks were fed in hard cardboard troughs and watered daily with distilled water in a glass fountain. The distilled water was stored in plastic bottles. Feed and water were supplied ad libitum. The basal diet used in these experiments contained the following ingredients expressed as grams per 100 gm: cerelose, 59.30; isolated soybean protein[2],

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TABLE 1

Zinc minerals and compounds used to test availability of zinc to the growing chick

Name and synonym	Formula	Per cent zinc
Sphalerite (zincblende)	ZnS	64.2[1]
Smithsonite (calamine, dry bone)	$ZnCO_3$	46.8[1]
Hemimorphite (calamine)	$2ZnO \cdot SiO_2 \cdot H_2O$	47.9[1]
Willemite (troosite, var. cont. Mn)	Zn_2SiO_4(+Mn)	46.7[1]
Franklinite	$(Fe, Mn, Zn, FeO_2)_2$	16.3[1]
Zincite (red zinc ore)	ZnO	65.3[1]
Sterling black and brown crude ore[2]		16.8[1]
Zinc oxide (technical grade)	ZnO	79.6[3]
Zinc sulfate (A. R. grade)	$ZnSO_4 \cdot 7H_2O$	22.7[3]
Zinc carbonate (A. R. grade)	$ZnCO_3$	52.1[3]
Zinc oxide (A. R. grade)	ZnO	80.3[3]
Zinc metal powder (dust)	Zn	100.0[3]

[1] Zinc content of samples determined by analysis conducted by the New Jersey Zinc Co., New York, New York.

[2] Mixture of franklinite, willemite, calcite and other minerals from Sterling Mine, Ogdensburg, New Jersey.

[3] Calculated zinc content from formula weights.

TABLE 2

Availability to chickens of the zinc in various zinc ores and compounds

Supplemental zinc	Experiment 1		Experiment 2		Experiment 3	
	Zinc source	Av. weight 2 weeks[1]	Zinc source	Av. weight 2 weeks[1]	Zinc source	Av. weight 2 weeks[1]
ppm		*gm*		*gm*		*gm*
0	$ZnSO_4 \cdot 7H_2O$, A.R.	84 ± 2.5	$ZnSO_4 \cdot 7H_2O$, A.R.	96 ± 2.6	$ZnSO_4 \cdot 7H_2O$, A.R.	81 ± 2.9
10	$ZnSO_4 \cdot 7H_2O$, A.R.	138 ± 3.8	$ZnSO_4 \cdot 7H_2O$, A.R.	136 ± 4.6	$ZnSO_4 \cdot 7H_2O$, A.R.	114 ± 3.5
20	$ZnSO_4 \cdot 7H_2O$, A.R.	162 ± 3.6	$ZnSO_4 \cdot 7H_2O$, A.R.	162 ± 3.3	$ZnSO_4 \cdot 7H_2O$, A.R.	136 ± 3.8
40	$ZnSO_4 \cdot 7H_2O$, A.R.	161 ± 3.0	$ZnSO_4 \cdot 7H_2O$, A.R.	171 ± 3.7	$ZnSO_4 \cdot 7H_2O$, A.R.	143 ± 4.6
10	Sphalerite	90 ± 3.2	Willemite	142 ± 3.3	Sterling black and brown crude ore	110 ± 2.6
20	Sphalerite	91 ± 2.7	Willemite	164 ± 3.9	Sterling black and brown crude ore	128 ± 3.2
10	Smithsonite	128 ± 2.6	Franklinite	103 ± 3.3	Zinc metal	117 ± 3.2
20	Smithsonite	159 ± 2.8	Franklinite	106 ± 4.1	Zinc metal	139 ± 4.3
10	Hemimorphite	135 ± 2.6	Zincite	129 ± 3.6	ZnO (technical)	120 ± 3.7
20	Hemimorphite	159 ± 3.0	Zincite	160 ± 3.2	ZnO (technical)	141 ± 3.5
10	ZnO, A.R.	130 ± 2.4	$ZnCO_3$, A.R.	137 ± 4.1		
20	ZnO, A.R.	161 ± 3.1	$ZnCO_3$, A.R.	161 ± 3.4		

[1] Three lots of 10 chicks each per treatment, mean ± standard error.

28.29; cellulose,[3] 3.0; corn oil, 3.0; DL-methionine, 0.69; glycine, 0.34; vitamin A concentrate (30,000 USP units/gm), 0.05; NaCl, 0.75; KCl, 0.60; $MgSO_4$, 0.255; *d*-α-tocopheryl acetate concentrate (20,000 I.U./lb.), 0.154; choline chloride concentrate (70.7%), 0.282; tricalcium phosphate N.F., 3.00; calcium carbonate A.R., 0.10; plus the following expressed as milligrams per 100 gm: inositol, 110.0; *p*-aminobenzoic acid, 11.0; calcium pantothenate, 2.20; niacin, 2.64; thiamine·HCl, 1.32; riboflavin, 1.32; pyridoxine·HCl, 0.66; folic acid, 0.44; menadione, 0.22; biotin, 0.44; vitamin B_{12}, 0.022; vitamin D_3 concentrate (15,000 ICU/gm), 13.332; $MnSO_4 \cdot H_2O$, 26.4; $FeSO_4 \cdot 7H_2O$, 11.0; $CuSO_4 \cdot 5H_2O$, 1.1; $CoCl_2 \cdot 6H_2O$, 1.1; $Na_2MoO_4 \cdot 2H_2O$, 0.11.

The biological assay type of experiment using chick growth as the criterion was used to evaluate the availability of zinc from 7 ores and 5 zinc compounds. The name, formula, and the percentage of zinc in each of the minerals and compounds tested are shown in table 1.

Zinc sulfate was chosen as the standard since it is a pure compound and is highly soluble in water. It was fed in the diet at three levels in order to obtain a standard growth response curve. The materials to be evaluated were fed in the diet at two levels.

RESULTS AND DISCUSSION

Three experiments were conducted to evaluate the various ores and compounds. The results of these experiments are shown in table 2. In the first experiment, only the zinc in sphalerite was found to be unavailable to the chick while smithsonite, hemimorphite and zinc oxide A. R. were all available sources of zinc when compared with zinc sulfate. In experiment 2, willemite, franklinite, zincite and zinc carbonate were tested. Of these 4 materials, only the zinc in franklinite was found to be relatively unavailable to the chick when compared to that in zinc sulfate. In experiment 3, the sterling black and brown crude ore, zinc metal and zinc oxide (technical grade) were evaluated. The zinc in sterling black and brown crude ore appeared to be less available than that in zinc sulfate. Zinc from zinc metal and zinc oxide was slightly more available than that from zinc sulfate.

One of the rather interesting observations was the low availability of zinc in franklinite. This mineral is composed of iron, manganese and zinc oxide. Results of the study reported here indicated that zinc in zinc oxide as a purified compound, technical grade compound, or a crude ore (zincite) is quite available. Therefore, it would appear that the crystal structure that results from the mixture of these metallic oxides prevents the zinc in franklinite from being available to the chicken.

SUMMARY

The results indicate that the zinc in zinc sulfate, willemite, zinc carbonate, zinc metal, zinc oxide (technical grade), smithsonite, hemimorphite, zinc oxide (A. R. grade) and zincite is relatively available to the young growing chicken. The zinc in sterling black and brown crude ore is of lower availability and that from sphalerite and franklinite is relatively unavailable.

ACKNOWLEDGMENTS

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[2] Archer-Daniels-Midland Company, Cincinnati, Ohio.

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

LITERATURE CITED

O'Dell, B. L., and J. E. Savage 1957 Symptoms of zinc deficiency in the chick. Federation Proc., 16: 394.

Studies with the Use of Co^{60}-Labeled Vitamin B_{12} on the Interrelationship of Choline and Vitamin B_{12} in Rats with Nutritional Edema[1]

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Earlier studies demonstrated that edema could be produced in rats by feeding a diet low in protein and devoid of choline, folacin and vitamin B_{12} (Alexander and Engel, '52; Alexander and Sauberlich, '57). The edema was prevented by choline, dimethylaminoethanol, betaine or methionine. Vitamin B_{12} was also partially effective, and its action was increased by the addition of folacin, which alone was without effect (Alexander and Sauberlich, '57).

The present investigation was undertaken to study further the metabolism of vitamin B_{12} in the edematous rat with the aid of Co^{60}-labeled vitamin B_{12}.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain were housed individually in wire-bottom cages in an air-conditioned room. Food and water were given ad libitum and the animals were weighed weekly. The composition of the basal diets used is given in table 1. Rats that received the edema-inducing diets were given an initial choline chloride supplement equal to 0.1% of the diet. This was reduced to 0.05% of the diet after two weeks and completely withdrawn two weeks later. This procedure was necessary in order to prevent deaths as a result of the development of hemorrhagic kidneys. Control animals received the same diet supplemented with choline chloride at a level of 0.2% of the diet for the entire experimental period. Other groups of animals received supplements either of vitamin B_{12}, or of vitamin B_{12} and folacin, or of choline, vitamin B_{12} and folacin (table 1, diets I to VI). An additional group received for a similar period a stock diet routinely fed rats of the breeding colony maintained at this laboratory. Hemoglobin determinations and serum electrophoresis analyses were made on the animals to note the onset of edema. Edema was observed in the present study only in rats fed diet no. II (no choline, table 1).

After a period of 22 weeks, rats fed the choline-free diet (diet II) developed edema. Upon the onset of edema, the rats usually failed to live more than a few days. At this point, Co^{60}-labeled vitamin B_{12}[2] (B_{12}*) of relatively high specific activity was administered to the experimental animals. A dose of 0.81 μC was administered to each animal subcutaneously, representing only 0.96 μg of vitamin B_{12}. The administered dose had an activity of 882,700 counts per minute, corrected, when measured in a Nuclear-Chicago Model DS-2 scintillation well counter.

After injection, the rats were placed individually in all-glass metabolism cages and urine and feces were collected separately for a 65-hour period. The animals were then sacrificed and tissues saved for radioactivity measurements. Some of the rats that received supplements were sacri-

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[2] The Co^{60}-labeled vitamin B_{12} was obtained from Abbott Laboratories, Oak Ridge, Tennessee and was used immediately upon receipt.

TABLE 1

Composition of experimental diets

(grams/kilogram of diet)

Ingredient	Diet no.[1]					
	I	II	III	IV	V	VI
Extracted peanut meal	150	150	150	150	150	150
Sucrose	576	578	576	578	578	571.5
Extracted casein	20	20	20	20	20	20
Salts[2]	50	50	50	50	50	50
Lard	190	190	190	190	190	190
Cod liver oil	10	10	10	10	10	10
L-Cystine	1	1	1	1	1	1
Inositol	1	1	1	1	1	1
Choline chloride	2	—	2	—	—	—
Betaine	—	—	—	—	—	6.5
Folacin, *mg*	(2)	—	—	—	(2)	—
Vitamin B_{12}, *μg*	(50)	—	—	(50)	(50)	—
α-Tocopherol acetate, *mg*	(100)	(100)	(100)	(100)	(100)	(100)
Ca pantothenate, *mg*	(30)	(30)	(30)	(30)	(30)	(30)
Niacin, *mg*	(25)	(25)	(25)	(25)	(25)	(25)
Thiamine, *mg*	(6)	(6)	(6)	(6)	(6)	(6)
Riboflavin, *mg*	(6)	(6)	(6)	(6)	(6)	(6)
Pyridoxine, *mg*	(6)	(6)	(6)	(6)	(6)	(6)
Menadione, *mg*	(5)	(5)	(5)	(5)	(5)	(5)
Biotin, *mg*	(0.5)	(0.5)	(0.5)	0.5)	(0.5)	(0.5)

[1] Values within parentheses indicate amount in milligrams or micrograms added at the expense of the entire diet.

[2] Salmon, W. D., J. Nutrition, 33: 155 (1947).

ficed 17 days after injection. The activity of the tissues was measured with the use of a scintillation well counter as previously indicated. Three rats were used for each time interval. As indicated in the tables, the variation in results between animals within a given group was generally reasonably low.

RESULTS

The distribution of activity of the administered vitamin B_{12}* in the various organs and tissues is presented in tables 2 to 4. Approximately 20 to 25% of the injected activity was found to be lost in the urine and feces during the first 65 hours. In each instance, over 70% of this activity loss was in the urine during the first 24-hour period. This relationship was noted as well with rats that had developed edema. Body size did not appear to influence the results since in supplementary experiments the amount of radioactivity and vitamin B_{12} injected was reduced to one-half the regular amount, but the relative proportions appearing in the tissues and excreta did not appear to be altered. Only rats fed diet IV (supplemented only with vitamin B_{12}) excreted smaller amounts of activity in the urine and feces during the initial 65-hour period.

The control rats, supplemented with choline, folacin and vitamin B_{12} (diet I; table 2), were found to have at the end of the 65-hour period 36.0% of the injected activity in the kidneys, 2.0% in the liver, 0.63% in the testicle, 0.37% in the heart, 0.33% in the pancreas, 0.27% in the spleen, and 0.27% in the lung (table 2). On the basis of concentration of activity (per cent of dose per gram of wet tissue), kidney was the highest, 18.5%; followed by adrenal, 2.1%; small intestine, 0.52%; spleen, 0.38%; pancreas, 0.35%; heart, 0.33%; testicle, 0.21%; lung, 0.20%; and liver, 0.16%. Smaller concentrations were found in the brain tissues, bone (femur), skin, muscle (*hind* thigh) and blood.

In the rats examined at the end of the 17-day period, remarkably high amounts of the administered activity were present in the kidneys (18.5%), liver (6.0%), pancreas (0.62%), testicle (0.55%), and heart (0.33%). Although the total amount of activity and the concentration of activity in the kidney at the end of the 17-day period was about one-half of that at 65

TABLE 2

The influence of diet on the distribution of subcutaneously administered vitamin B_{12}-Co^{60} in the adult rat

Tissue	Distribution of administered vitamin B_{12}-Co^{60}							
	Diet I; with choline, folacin and vitamin B_{12}				Stock diet			
	% of total dose at:		% of dose/gm tissue		% of total dose at:		% of dose/gm tissue	
	65 hrs.	17 days	65 hrs.	17 days	65 hrs.	17 days	65 hrs.	17 days
Kidney	36.05 ± 4.57[1]	18.15 ± 1.92	18.50 ± 3.37	7.05 ± 0.60	29.48 ± 0.05	17.25 ± 0.30	9.94 ± 0.11	9.66 ± 0.25
Liver	2.03 ± 0.17	6.00 ± 0.28	0.16 ± 0.01	0.23 ± 0.02	3.15 ± 0.55	6.13 ± 0.21	0.21 ± 0.05	0.71 ± 0.03
Adrenal	0.07 ± 0.02	—	2.10 ± 0.40	—	0.09 ± 0.02	—	1.72 ± 0.28	—
Spleen	0.27 ± 0.06	0.10 ± 0.02	0.38 ± 0.02	0.14 ± 0.02	0.38 ± 0.01	0.12 ± 0.01	0.52 ± 0.06	0.40 ± 0.05
Pancreas	0.33 ± 0.05	0.62 ± 0.11	0.35 ± 0.03	0.56 ± 0.13	0.80 ± 0.07	1.34 ± 0.06	0.53 ± 0.12	1.13 ± 0.08
Heart	0.37 ± 0.05	0.33 ± 0.02	0.33 ± 0.02	0.27 ± 0.02	0.49 ± 0.01	0.40 ± 0.01	0.39 ± 0.01	0.47 ± 0.02
Lung	0.27 ± 0.01	0.13 ± 0.01	0.20 ± 0.01	0.08 ± 0.02	0.42 ± 0.05	0.28 ± 0.01	0.20 ± 0.01	0.21 ± 0.02
Testicle	0.63 ± 0.01	0.55 ± 0.08	0.21 ± 0.01	0.16 ± 0.01	0.82 ± 0.01	—	0.24 ± 0.01	—
Brain	0.19 ± 0.01	0.21 ± 0.02	0.10 ± 0.01	0.11 ± 0.01	0.24 ± 0.02	0.28 ± 0.03	0.13 ± 0.01	0.16 ± 0.02
Small intestine[2]	—	—	0.52 ± 0.03	0.10 ± 0.01	—	—	0.55 ± 0.05	0.21 ± 0.03
Blood	—	—	0.011 ± 0.001	0.003 ± 0.001	—	—	0.013 ± 0.001	0.006 ± 0.001
Muscle[3]	—	—	0.050 ± 0.006	0.031 ± 0.005	—	—	0.060 ± 0.001	0.052 ± 0.002
Skin	—	—	0.057 ± 0.004	0.018 ± 0.003	—	—	0.085 ± 0.005	0.054 ± 0.005
Bone[3]	—	—	0.050 ± 0.012	—	—	—	0.061 ± 0.006	0.044 ± 0.010
Urine[4]	21.92 ± 2.81	—	—	—	18.13 ± 0.06	—	—	—
Feces[4]	4.41 ± 1.51	—	—	—	3.09 ± 0.90	—	—	—
Dose retained:	73.67%	—	—	—	78.78%	—	—	—

[1] Standard deviation.

[2] Portion approximately six inches in length, starting from the duodenal region.

[3] Femoral muscles and bone.

[4] For the total 65-hour period.

hours, that of the liver and pancreas had increased during this period. The concentration of activity in the small intestine, spleen and lungs, however, decreased, while that in the heart, brain, and testicle remained about the same. The tissues appeared normal, with the exception that the livers occasionally were slightly fatty and pale.

The distribution of the administered vitamin B_{12}* in rats maintained on a stock diet (table 2) was similar to the above control rats receiving supplements of choline, folacin and vitamin B_{12}. However, the animals fed the stock diet had somewhat higher amounts of activity in the liver and lower amounts in the kidneys at the 65-hour period than the control rats. At the end of the 17-day period, the stock rats had generally higher amounts or concentrations in the tissues and organs analyzed than the control rats. These concentrations or amounts, with the exception of kidney tissue, were usually considerably lower than those noted in the tissues of rats fed diets free of or supplemented with choline or betaine (tables 3 and 4).

Rats with edema (diet II; no choline, vitamin B_{12} or folacin) were found to have at the end of the 65-hour period 22.3% of the injected activity in the kidneys, 9.2% in the liver, 1.4% in the heart, 0.97% in the lung, 0.96% in the spleen and 0.83% in the pancreas (table 3). On the basis of concentration of activity per gram of tissue, kidney was the highest, 15.0%; followed by small intestine, 2.1%; liver, 1.9%; adrenal, 1.9%; heart, 1.3%; spleen, 1.1%; and pancreas, 1.0%. Smaller concentrations were found in the lung, testicle and brain tissues. Small, but significant, concentrations were also found in bone, skin, muscle and blood. Thus, rats with edema, in comparison with control animals (diet I, table 2), had lower amounts and concentrations of the administered activity in the kidneys, but considerably higher amounts and concentrations in the liver, spleen, pancreas, heart, lung, brain and small intestine. No data were obtained for the 17-day period on the edematous rats since they lived only a few days after onset of the condition.

The edematous rats upon sacrifice had generalized edema, ascites, and hydrothorax. The liver was pale, fatty and cirrhotic, with nodules up to 2 mm in diameter over the surface. The pancreas was pale and edematous and the heart and kidneys frequently had lesions and enlargement. The spleen was also enlarged, but the testicles were smaller than normal.

Rats supplemented only with choline (diet III; table 3) also had a high percentage of the administered activity in the kidneys (19.8%), but less was found in the liver (5.6%), spleen (0.54%), heart (0.63%), pancreas (0.70%) and lung (0.65%) when compared with the edematous rats (diet II). On the basis of concentration of activity per gram of tissue, kidney was the highest, followed by adrenal, small intestine, spleen, pancreas, heart, lung and liver. The liver was thus considerably lower in activity in comparison with other tissues when choline was fed to the rats.

In the rats examined at the end of the 17-day period, remarkably high amounts of the administered activity were present in the kidneys (11.7%), liver (9.4%), pancreas (1.7%) and heart (0.76%). Although the total amount of activity and the concentration of activity in the kidney at the end of the 17-day period was about one-half of that at 65 hours, that of the liver and pancreas had increased during this period. The concentration of activity in the small intestine, spleen and lungs, however, decreased, while that in the heart, testicle and brain remained about the same. The tissues from rats supplemented with choline appeared normal, except for the liver, which was pale and fatty. No evidence of edema occurred.

Rats supplemented with betaine instead of choline were found to have essentially the same distribution of activity of the administered vitamin B_{12}* as of those supplemented with choline (diet VI; table 4). This was true at both the 65-hour period and the 17-day period. No edema was present in the animals and the tissues were normal in appearance, except for the liver, which was pale and fatty.

Supplementation of the diet only with vitamin B_{12} (50 μg per kg of diet) appeared to influence the retention and distribution in the tissues of the injected vitamin B_{12}* activity (diet IV; table 3). Only about

TABLE 3

The influence of diet on the distribution of subcutaneously administered vitamin B_{12}-Co^{60} in the adult rat

Tissue	Distribution of administered vitamin B_{12}-Co^{60}							
	Diet II; no choline		Diet III; with choline				Diet IV; with vitamin B_{12}	
	% of total dose at:	% of dose/ gm tissue	% of total dose at:		% of dose/ gm tissue		% of total dose at:	% of dose/ gm tissue
	65 hrs.	65 hrs.	65 hrs.	17 days	65 hrs.	17 days	65 hrs.	65 hrs.
Kidney	22.29 ± 6.82[1]	14.97 ± 6.07	19.78 ± 2.28	11.75 ± 3.13	8.78 ± 2.04	4.37 ± 0.60	52.35 ± 2.62	27.53 ± 4.07
Liver	9.23 ± 2.16	1.88 ± 0.18	5.60 ± 0.69	9.39 ± 0.21	0.43 ± 0.08	0.56 ± 0.01	2.43 ± 0.06	0.14 ± 0.01
Adrenal	0.10 ± 0.02	1.88 ± 0.17	0.09 ± 0.02	—	2.15 ± 0.26	—	0.08 ± 0.02	1.94 ± 0.17
Spleen	0.96 ± 0.19	1.14 ± 0.18	0.54 ± 0.01	0.32 ± 0.02	0.97 ± 0.07	0.56 ± 0.01	0.30 ± 0.04	0.32 ± 0.02
Pancreas	0.83 ± 0.07	1.04 ± 0.15	0.70 ± 0.12	1.73 ± 0.27	0.77 ± 0.08	2.06 ± 0.23	0.37 ± 0.12	0.28 ± 0.04
Heart	1.39 ± 0.19	1.28 ± 0.17	0.63 ± 0.04	0.76 ± 0.01	0.59 ± 0.03	0.68 ± 0.02	0.33 ± 0.01	0.28 ± 0.04
Lung	0.97 ± 0.16	0.80 ± 0.09	0.65 ± 0.05	0.50 ± 0.17	0.46 ± 0.03	0.28 ± 0.05	0.33 ± 0.03	0.28 ± 0.08
Testicle	0.40 ± 0.06	0.44 ± 0.01	0.73 ± 0.04	0.53 ± 0.15	0.23 ± 0.01	0.24 ± 0.01	0.65 ± 0.07	0.20 ± 0.01
Brain	0.57 ± 0.04	0.35 ± 0.04	0.39 ± 0.06	0.49 ± 0.03	0.24 ± 0.04	0.26 ± 0.01	0.20 ± 0.07	0.11 ± 0.01
Small intestine	—	2.07 ± 0.30	—	—	1.01 ± 0.04	0.22 ± 0.02	± 0.01	0.45 ± 0.08
Blood	—	0.08 ± 0.01	—	—	0.02 ± 0.004	0.009 ± 0.001	—	0.012 ± 0.001
Muscle	—	0.12 ± 0.01	—	—	0.09 ± 0.004	0.054 ± 0.002	—	0.040 ± 0.001
Skin	—	0.20 ± 0.03	—	—	0.11 ± 0.02	0.100 ± 0.001	—	0.059 ± 0.001
Bone	—	0.31 ± 0.04	—	—	0.13 ± 0.02	—	—	0.062 ± 0.012
Urine[2]	19.14 ± 2.31	—	19.38 ± 0.84	—	—	—	11.55 ± 0.61	—
Feces[2]	2.89 ± 0.85	—	2.44 ± 0.73	—	—	—	1.19 ± 0.29	—
Dose retained:	77.97%		78.18%				87.26%	

[1] Standard deviation.

[2] For the total 65-hour period.

TABLE 4

The influence of diet on the distribution of subcutaneously administered vitamin B_{12}-Co^{60} in the adult rat

Tissue	Distribution of administered vitamin B_{12}-Co^{60}							
	Diet V; with folacin and vitamin B_{12}				Diet VI; with betaine			
	% of total dose at:		% of dose/gm tissue		% of total dose at:		% of dose/gm tissue	
	65 hrs.	17 days	65 hrs.	17 days	65 hrs.	17 days	65 hrs.	17 days
Kidney	35.66 ± 3.93[1]	22.97 ± 1.87	16.64 ± 2.72	9.70 ± 1.07	20.82 ± 3.44	12.13 ± 0.77	10.88 ± 1.92	5.09 ± 0.23
Liver	1.89 ± 0.36	3.31 ± 0.06	0.17 ± 0.03	0.24 ± 0.01	4.17 ± 0.09	8.16 ± 0.62	0.37 ± 0.05	0.65 ± 0.06
Adrenal	0.06 ± 0.01	—	1.28 ± 0.09	—	0.13 ± 0.01	—	2.66 ± 0.36	—
Spleen	0.18 ± 0.01	0.12 ± 0.01	0.31 ± 0.04	0.19 ± 0.01	0.49 ± 0.05	0.43 ± 0.03	0.85 ± 0.05	0.62 ± 0.06
Pancreas	0.36 ± 0.06	0.73 ± 0.01	0.32 ± 0.01	0.57 ± 0.07	0.90 ± 0.06	1.94 ± 0.18	0.84 ± 0.01	1.76 ± 0.10
Heart	0.40 ± 0.01	0.38 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.68 ± 0.16	0.71 ± 0.07	0.70 ± 0.21	0.63 ± 0.04
Lung	0.21 ± 0.02	0.16 ± 0.01	0.18 ± 0.02	0.11 ± 0.01	0.60 ± 0.01	0.42 ± 0.01	0.43 ± 0.03	0.29 ± 0.01
Testicle	0.59 ± 0.03	0.55 ± 0.04	0.17 ± 0.01	0.15 ± 0.01	0.86 ± 0.14	0.94 ± 0.07	0.28 ± 0.02	0.28 ± 0.01
Brain	0.17 ± 0.01	0.19 ± 0.01	0.09 ± 0.002	0.11 ± 0.004	0.38 ± 0.02	0.40 ± 0.08	0.20 ± 0.03	0.25 ± 0.01
Small intestine	—	—	0.55 ± 0.08	0.13 ± 0.01	—	—	1.23 ± 0.18	0.24 ± 0.01
Blood	—	—	0.010 ± 0.001	0.005 ± 0.001	—	—	0.02 ± 0.002	0.009 ± 0.002
Muscle	—	—	0.045 ± 0.001	0.042 ± 0.003	—	—	0.11 ± 0.008	0.056 ± 0.010
Skin	—	—	0.048 ± 0.011	0.024 ± 0.002	—	—	0.12 ± 0.020	0.073 ± 0.005
Bone	—	—	0.042 ± 0.001	—	—	—	0.11 ± 0.010	—
Urine[2]	22.80 ± 0.70	—	—	—	18.35 ± 2.64	—	—	—
Feces[2]	2.21 ± 0.32	—	—	—	2.35 ± 1.05	—	—	—
Dose retained:	74.99%				79.30%			

[1] Standard deviation.

[2] For the total 65-hour period.

13% of the dose was found in the urine and feces at the end of the 65-hour period. The kidneys accounted for 52.3% of the activity administered and only 2.4% was present in the liver. The amounts in the other organs were also low when compared with rats fed diet II (no choline) or diet III (with choline). The concentration of activity per gram of tissue was also very high in the kidney (27.5% per gm) and low in the liver (0.14% per gm). In general, the amounts and concentrations of activity found in the tissues were similar to those noted in the control animals (diet I, table 2). The tissues of the vitamin B_{12} supplemented rats appeared normal, except for the liver, which was pale, fatty, enlarged, and possessed some regeneration areas.

When the rats received supplements of folacin in addition to vitamin B_{12} (diet V; table 4), the amount of administered activity retained at the 65-hour period was reduced (75.0%) as was the amount in the kidneys (35.7%). The amount of activity in the other tissues measured at the 65-hour period was found to be about the same as in tissues from similarly treated rats receiving supplements of only vitamin B_{12} or control animals receiving supplements of choline, folacin and vitamin B_{12}.

At the end of the 17-day period, the amount of the administered activity in the kidneys had dropped from 35.6% at 65 hours to 23%, while the amount of activity present in the liver had increased from 1.9% to 3.3%. The activity in the pancreas also increased considerably, while that in the other tissues (total or per gram) either decreased or remained essentially the same over this period of time. The tissues appeared normal, except for the liver, which was somewhat fatty and pale.

DISCUSSION

Results of the present studies indicate an association of vitamin B_{12} with choline as was first observed by Schaefer et al. ('49). This may be noted from the fact that the rats fed choline retained less of the radioactive vitamin B_{12} (B_{12}*) in their organs than did the choline-deficient animals. The effect was particularly noticeable for the liver. Choline may reduce the amount of vitamin B_{12} needed by the rat and thus permit greater retention of the original stores of the vitamin in the tissues.

In this respect it is of interest that the kidneys appeared to act as a storage site of vitamin B_{12}, at least initially. Rats fed diets supplemented with vitamin B_{12} retained more of the vitamin B_{12}* in the kidneys than did animals receiving diets with or without supplements of choline or betaine. However, in most species of animals studied, the highest concentrations of vitamin B_{12} are in the liver, followed by the kidney, pancreas, spleen, heart, brain and lung (Scheid and Schweigert, '54). Other investigations with the rat have also revealed the kidneys to be a very active site of storage of administered vitamin B_{12}* (Barbee and Johnson, '51; Chow et al., '51; Rosenblum et al., '52). More recently, Miller et al. ('56) have noted that in the hamster, mouse and guinea pig the highest amounts of injected vitamin B_{12}* were located in the liver, while in the normal rat more was located in the kidneys than in other organs.

Several investigators (Barbee and Johnson, '51; Rosenblum et al., '52) have noted that, in the rat, the urinary excretion of injected Co^{60}-labeled vitamin B_{12} was greater than that appearing in the feces. However, Miller et al. ('56) have noted the reverse condition. Although significant amounts of activity appeared in the feces in the present study, considerably more was eliminated in the urine. Moreover, the proportions did not appear to be the result of the amounts of vitamin B_{12} injected since in a supplementary study, the amount of radioactivity and of vitamin B_{12} was reduced by 50% (0.48 μg of vitamin B_{12}*). In these experiments, similar proportions, but smaller amounts, appeared in the urine and feces. However, of interest was the observation that the small intestine possessed relatively high amounts of radioactivity. The activity in this tissue appeared to disappear more rapidly than was noted for the other tissues. This may indicate an association of the small intestine in the metabolism or excretion of vitamin B_{12}.

It is apparent from the high amounts of activity present 17 days after injection that vitamin B_{12} is retained quite firmly by

the tissues. In this regard it is interesting to note the increase in radioactivity observed in the liver, pancreas, brain and certain other tissues at the end of this period. For instance, the amount of activity in the liver of rats supplemented only with choline was now nearly equal to that present in the kidneys. This would indicate that vitamin B_{12} in its metabolism is transferred from the kidney to the liver and other sites. The presence of vitamin B_{12} in the diet at the levels used in this study did not appear to influence markedly the rate of transfer or removal of activity from the organs or tissues. Harte et al. ('53) found similar, although less marked, changes with time in the storage of Co^{60}-labeled vitamin B_{12} in the liver, kidney, pancreas and spleen of rats fed a stock diet.

The pancreas is apparently also a site of considerable activity of vitamin B_{12} metabolism as may be indicated by the increase in radioactivity at the end of the 17-day period. Rats maintained on choline-free diets, and particularly animals with edema, were observed to have a pale and edematous pancreas, while this organ appeared normal in rats receiving supplements of choline or of vitamin B_{12}.

As was previously observed, rats with edema invariably were found to have fatty infiltration and cirrhosis of the liver. The presence of choline or of vitamin B_{12} and folacin in the diet prevented the cirrhosis and markedly reduced or averted the infiltration of fat. The cirrhotic, fatty liver may fail to function properly in the maintenance of normal serum protein levels and thereby initiate the edematous condition. However, this abnormal condition of the liver did not appear to depress its retention of the administered vitamin B_{12}*. Similarly, several other organs in the edematous rat showed pathological changes, but readily retained the radioactivity.

In other unpublished studies, no success has been achieved in producing edema in rats by feeding diets adequate in choline but low in certain essential amino acids. In these experiments, no liver damage was observed.

SUMMARY

Edema was produced in rats fed a diet low in protein and devoid of choline, vitamin B_{12} and folacin. The edema was prevented by dietary supplements of either choline, betaine or vitamin B_{12} or a combination of choline, folacin, and vitamin B_{12}.

The distribution of subcutaneously injected Co^{60}-labeled vitamin B_{12} was studied in rats with experimental nutritional edema. Approximately 75% of the administered activity was retained by the animals at the end of a 65-hour equilibration period. The kidneys were the primary site of retention of activity, followed by the liver, pancreas, heart, lung and spleen, with smaller amounts in the other tissues. Relatively high concentrations of activity were noted in the adrenals and small intestine.

Dietary supplements of choline or betaine reduced the retention of activity by the organs, while supplements either of vitamin B_{12}, or of vitamin B_{12} and folacin, or of choline, vitamin B_{12}, and folacin increased the retention of activity by the kidneys, but markedly reduced the amounts in the other organs.

After a period of 17 days, the amount of radioactivity in the small intestine was reduced considerably and reduced somewhat in the kidneys, while the amount in the liver and pancreas was increased noticeably.

LITERATURE CITED

Alexander, H. D., and R. W. Engel 1952 The importance of choline in the prevention of nutritional edema in rats fed low-protein diets. J. Nutrition, *47:* 361.

Alexander, H. D., and H. E. Sauberlich 1957 The influence of lipotropic factors on the prevention of nutritional edema in the rat. Ibid., *61:* 329.

Barbee, K. W., and B. C. Johnson 1951 Metabolism of radioactive vitamin B^{12} by the rat. Proc. Soc. Exp. Biol. Med., *76:* 720.

Chow, B. F., C. Rosenblum, R. H. Silber, P. T. Woodbury, R. Yamamoto and C. A. Lang 1951 Oral administration of vitamin B_{12} containing cobalt-60 to rats. Ibid., *76:* 393.

Harte, R. A., B. F. Chow and L. Barrows 1953 Storage and elimination of vitamin B_{12} in the rat. J. Nutrition, *49:* 669.

Miller, A., G. Gaull, J. F. Ross and H. M. Lemon 1956 Tissue distribution of parenteral Co^{60} vitamin B_{12} in mouse, hamster, rat and guinea pig. Proc. Soc. Exp. Biol. Med., *93:* 33.

Rosenblum, C., B. F. Chow, G. P. Condon and R. Yamamoto 1952 Oral versus parenteral administration of Co^{60}-labeled vitamin B_{12}. J. Biol. Chem., *198:* 915.

Schaefer, A. E., W. D. Salmon and D. R. Strength 1949 Interrelationship of vitamin B_{12} and choline. 1. Effect on hemorrhagic kidney syndrome in the rat. Proc. Soc. Exp. Biol. Med., *71:* 193.

Scheid, H. E., and B. S. Schweigert 1954 Vitamin B_{12} content of organ meats. J. Nutrition, *53:* 419.

Inhibition of the Amino Acid-Sugar Reaction

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Amino acids and reducing sugars react to form brown pigments through the well-known "browning reaction" (Hodge, '53). Foodstuffs and nutritional supplements containing these materials may deteriorate in quality as the reaction proceeds. Previous studies from this laboratory (Overby and Frost, '52) have shown that the nutritive value of fibrin hydrolysates with dextrose is related inversely to the color of the solution. Various sulfites inhibit the development of color and delay loss of amino acid feeding value. In the present studies the objectives were (a) to compare potassium metabisulfite, sodium hydrosulfite, and sodium formaldehyde sulfoxylate in their capacity to inhibit color formation in the following aqueous solutions: fibrin hydrolysate with glucose, fibrin hydrolysate with fructose, glycine with glucose, and glycine with fructose; (b) to follow loss of nutritive value of the hydrolysates with hexose by rat repletion assay.

METHODS

The fibrin hydrolysate was a commercial preparation from beef fibrin, containing about two-thirds free amino acids and one-third small peptides. Glucose, fructose, and glycine were U.S.P. grade chemicals. Four solutions were prepared as follows:

1. Eighty grams of glycine and 200 gm of glucose were dissolved in water and diluted to 2000 ml.
2. Similarly, a solution of 80 gm of glycine and 200 gm of fructose was prepared.
3. Two hundred grams of glucose was dissolved in a quantity of fibrin hydrolysate so that, when diluted to 2000 ml, the total nitrogen was 6.5 mg/ml.
4. A solution similar to (3) was prepared using 200 gm of fructose with fibrin hydrolysate.

The solutions were saturated with CO_2, and 0.400 gm of cysteine hydrochloride was added to each 2000 ml portion. The final solutions were divided into 500 ml portions and placed in closed bottles under CO_2 atmosphere. To each of three bottles of the 4 solutions described, there was added one of the following inhibitors: potassium metabisulfite, 0.720 gm/liter; sodium hydrosulfite, 0.565 gm/liter; and sodium formaldehyde sulfoxylate, 1.0 gm/liter. No inhibitor was added to the 4th bottle of each series. All solutions were then autoclaved at 110°C for 20 minutes and allowed to cool to room temperature in the opened autoclave. A sample was removed from each bottle, diluted with 4 volumes of water, and the optical density measured at 385 mu with a Beckman Model DU spectrophotometer.

For the nutritional studies, additional solutions of fibrin hydrolysate with glucose or with fructose were prepared as above with sodium formaldehyde sulfoxylate. After sterilization at 110°C for 10 minutes they were used directly or further treated in one of the following ways before testing:

1. Stored at 25°C for 15 months.
2. Stored at 4, 25 and 40°C for 4.5 months.
3. Autoclaved 15 minutes at 121°C.
4. Autoclaved 30 minutes at 121° C.

Nutritive value of the browned solutions was determined by the rat repletion method of Frost and Sandy ('48). Assays were carried out for 5 or 12 days at 0.24 gm nitrogen per rat per day. A commercial fibrin hydrolysate without carbohydrate[1] was used as a standard control.

RESULTS

The results of the optical density measurements on the 12 solutions autoclaved

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[1] Aminosol®, Abbott Laboratories, North Chicago, Illinois.

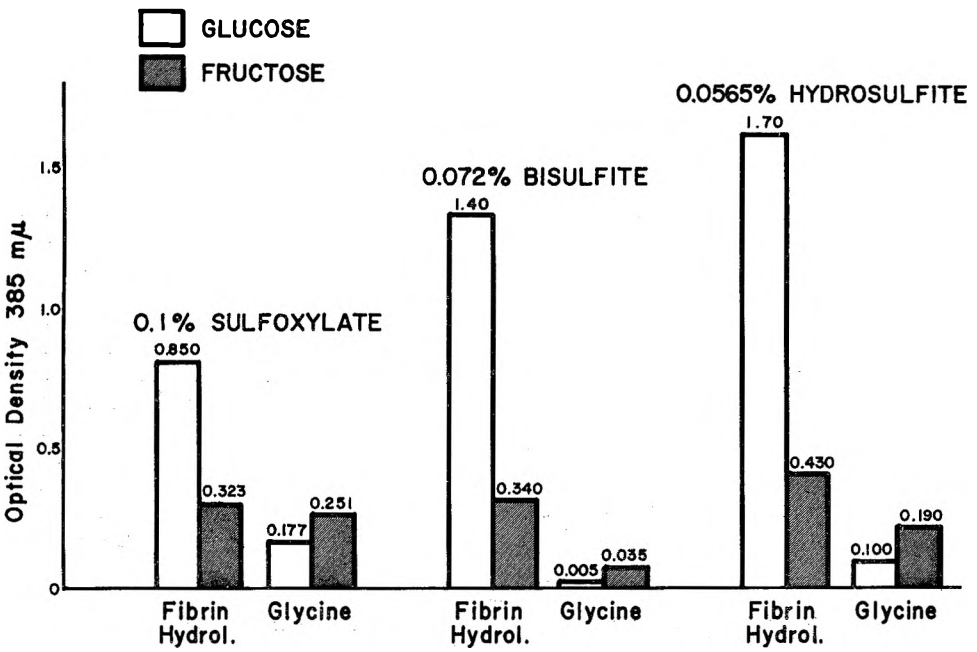

Fig. 1 Comparison of various sulfites as inhibitors of browning of fructose or glucose with glycine or fibrin hydrolysate. All solutions heated at 110°C for 20 minutes, then diluted 1 to 5. Optical densities of the solutions without inhibitors were: hydrolysate + glucose, 1.8; hydrolysate + fructose, 0.830; glycine + glucose, 0.449; glycine + fructose, 0.790.

with inhibitors are shown in figure 1. The following values were found on the 4 solutions autoclaved without inhibitors: fibrin hydrolysate with glucose, 1.8; fibrin hydrolysate with fructose, 0.830; glycine with glucose, 0.449; glycine with fructose, 0.790. It will be seen that each of the sulfite compounds inhibited color formation in all solutions.

Figure 1 makes three comparisons, as follows:

1. *Comparison of glucose and fructose with glycine or fibrin hydrolysate.* When the inhibitor was sodium formaldehyde sulfoxylate, color formation was considerably less with fructose-fibrin hydrolysate (0.323) than with glucose-fibrin hydrolysate (0.850). This difference between fructose and glucose solutions with mixtures of pure amino acids was observed by Ellingson, Mueller and Kemmerer ('54). In contrast to glucose (0.177), fructose reacted more rapidly with glycine (0.251). These same relationships were found with the other inhibitors tested.

2. *Comparison of inhibitors in reaction of fructose with glycine or fibrin hydrolysate.* With fructose-fibrin hydrolysate solutions sodium formaldehyde sulfoxylate was most effective (0.323), followed by potassium metabisulfite (0.340), and sodium hydrosulfite (0.430). Although these differences in optical densities were small, the colors of the undiluted solutions were easily distinguished visually, and were always in the same order. With fructose-glycine solutions practically the reverse picture was observed: sodium formaldehyde sulfoxylate was least effective (0.251), sodium hydrosulfite was better (0.190) and potassium metabisulfite was best (0.035).

3. *Comparison of inhibitors in reaction of glucose with glycine or fibrin hydrolysate.* The order of effectiveness of the inhibitors was not different from that found with fructose solutions, but all glucose-fibrin hydrolysate solutions showed more color and glucose-glycine solutions less color than the corresponding solutions with fructose. With fibrin hydrolysate-glucose, sodium formaldehyde sulfoxylate was most effective (0.85), followed by potassium metabisulfite

(1.4), and sodium hydrosulfite (1.7). The reaction between glucose and glycine was inhibited most by potassium metabisulfite (0.005), next by sodium hydrosulfite (0.100), and least by sodium formaldehyde sulfoxylate (0.177).

Comparison of nutritive value of fibrin hydrolysate with 10% glucose and 10% fructose after 15 months at 25°C

Table 1 shows the results of the rat repletion assays. A gain of 6.1 gm was obtained with the 10% glucose solution, 15.6 gm with the 10% fructose solution, and 19.8 gm with a control solution lacking carbohydrate. Sodium formaldehyde sulfoxylate was used in the solutions containing glucose and fructose. The color of the solutions was inversely related to the weight gain.

Stability of fibrin hydrolysate with fructose at graded temperatures

The above results indicated that the fibrin hydrolysate-fructose solution with sodium formaldehyde sulfoxylate was the most stable combination with respect to the browning reaction and showed but little loss of nutritive value after 15 months at 25°C. This solution was then tested under various conditions of storage and heat treatment with results shown in table 2. In experiment 1, fibrin hydrolysate with fructose after storage at 40°C for 4.5 months showed about 10% loss in nutritive value by actual weight gain, but statistically there were no differences in the gains for the 4 groups. In the second ex-

TABLE 1

Nutritive value of fibrin hydrolysate after 15 months at 25°C (Solutions with carbohydrate contained sodium formaldehyde sulfoxylate, 1 gm/liter).

	12-day rat repletion response[1]		
	Gain	Range	S.E.[2]
	gm	*gm*	*gm*
Standard[3]	19.8	16–22	± 0.25
Fibrin hydrolysate (5%) + fructose (10%)	15.6	4–22	± 2.1
Fibrin hydrolysate (5%) + glucose (10%)	6.1	0–12	± 1.4

[1] Eight rats per group; 0.24 mg N/rat/day.

[2] Standard error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[3] Response given by a standard hydrolysate without carbohydrate (Aminosol ®).

TABLE 2

Effect of graded temperatures on the nutritive value of fibrin hydrolysate (5%) with fructose (10%) and sodium formaldehyde sulfoxylate (1 gm/liter)

Treatment after sterilization at 110°C for 10 minutes			Average 5-day rat repletion response[1]		
Temp.	Time		Gain	Range	S.E.[2]
°C			*gm*	*gm*	*gm*
		Experiment 1			
4°	4.5 months		22.6	12–34	± 2.4
25°	4.5 months		26.2	22–34	± 1.6
40°	4.5 months		20.0	14–30	± 1.8
	Standard[3]		21.9	20–24	± 0.2
		Experiment 2			
None	—		18.0	16–19	± 0.12
121°	15 minutes		16.8	12–20	± 1.0
121°	30 minutes		13.7	12–18	± 0.26
	Standard[3]		16.2	12–22	± 1.25

[1] Eight rats per group; 0.24 mg N/rat/day.

[2] Standard error, see footnote 2, table 1.

[3] Response given by a standard hydrolysate without carbohydrate (Aminosol ®).

periment an accelerated rate of browning was produced by heat treatment. Four solutions were compared as shown. Autoclaving at 121°C for 15 minutes appeared to cause little or no loss of nutritive value. After 30 minutes at this temperature definite loss was observed.

DISCUSSION

The observations on the relative inhibitory capacities of bisulfite, hydrosulfite, and formaldehyde sulfoxylate indicate some basic differences in the reactions leading to color formation in reducing sugar-glycine solutions as compared to reducing sugar-fibrin hydrolysate solutions. These three sulfite compounds represented successive levels of oxidation-reduction potential. In the case of the model system of glycine-carbohydrate, bisulfite (the most highly oxidized compound) showed the greatest inhibitory capacity on an equimolar basis. For the complex mixture of fibrin hydrolysate-carbohydrate, sodium formaldehyde sulfoxylate (the least highly oxidized compound) was the most potent inhibitor. The picture is further complicated by the reversal of reactivity of glucose and fructose with glycine and fibrin hydrolysate.

These experiments suggest that the degree of inhibition of color development in fibrin hydrolysate-carbohydrate solutions is closely related to the preservation of nutritive value as measured by the rat repletion assay. Solutions of fructose, which showed a slower rate of browning, showed a slower rate of nutritional deterioration. Initial autoclaving caused only slight reduction in nutritive value. There appeared to be some decrease in rat repletion response after extended periods of storage at room temperature or higher.

It is difficult to assess the extent to which the initial amino acid-sugar reactions affect the biological efficacy of a particular mixture. Friedman and Kline ('50) indicated that fluorescence may be the earliest physical evidence of complex formation in hydrolysates with glucose. This reaction appeared to precede color development or loss of nutrient value found by the rat assay (Overby and Frost, '52). Significantly, Christensen et al. ('55, '56) showed prerenal rejection of the reaction products of partial protein hydrolysates with glucose when given intravenously to humans. Unpublished studies with dogs in this laboratory have revealed 13 to 19% loss in ability to maintain nitrogen balance when heat darkened hydrolysate-glucose solutions were administered intravenously.

It may be that amino acids in some intermediate "browning reaction" products are in part available when given orally to rats, but are not utilized when given intravenously to humans and dogs. Thus the rat repletion method used in these experiments may not give a critical evaluation of early loss of nutritive value. The method, now official in U.S.P. XV ('55), is used in experiments now underway to establish the rate of nutritive loss of various protein hydrolysates with glucose.

These experiments show that the appearance of dark colored reaction products may be delayed in amino acid-sugar solutions by using various sulfites as inhibitors. The addition of sodium formaldehyde sulfoxylate to fructose-hydrolysate mixtures gives the most stable combination. However, the total amino acid nutritive value inevitably lessens with temperature and time. A more critical biological test is needed to establish the extent of early nutritive loss before browning occurs.

SUMMARY

The browning reaction for glucose with fibrin hydrolysate proceeds more rapidly than for fructose with fibrin hydrolysate. However, fructose reacts more rapidly with glycine than does glucose.

Inhibitors of the chromogenic reaction between glucose or fructose and fibrin hydrolysate may be grouped as follows in decreasing order of effectiveness: sodium formaldehyde sulfoxylate, potassium metabisulfite, and sodium hydrosulfite. For glycine with either hexose the decreasing order of effectiveness is: potassium metabisulfite, sodium hydrosulfite and sodium formaldehyde sulfoxylate.

Fructose solutions with fibrin hydrolysate lose nutritive value, measured by rat repletion assay, less rapidly during storage than do corresponding glucose solutions. Although fructose solutions withstand ordinary autoclaving, nutritive value is diminished at 121°C for 30 minutes.

ACKNOWLEDGMENT

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LITERATURE CITED

Christensen, H. N., P. B. Wilbur, B. A. Coyne and J. H. Fisher 1955 Effects of simultaneous or prior infusion of sugars on the fate of infused protein hydrolysates. J. Clin. Invest., *34:* 86.

Christensen, H. N. 1956 Some aspects of parenteral amino acid nutrition. Univ. of Mich. Med. Bull., *22:* 67.

Ellingson, R. C., A. J. Mueller and K. S. Kemmerer 1954 Differences between fructose and glucose in the browning reaction. Federation Proc., *13:* 204.

Friedman, L., and O. L. Kline 1950 The amino acid-sugar reaction. J. Biol. Chem., *184:* 599.

Frost, D. V., and H. R. Sandy 1948 Assay of liquid protein hydrolysates in protein-depleted rats. Ibid., *175:* 635.

Hodge, J. E. 1953 Chemistry of browning reactions in model systems. J. Agr. Food Chem., *1:* 928.

Overby, L. R., and D. V. Frost 1952 The effects of heat on the nutritive value of protein hydrolysates with dextrose. J. Nutrition, *46:* 539.

Pharmacopoeia of the United States. 15th Revision 1955 Mack Publishing Co., Easton, Pa., pp. 882–883 and Supplement, p. 591

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