Isolation and Nature of an Unidentified Growth Factor(s) in Condensed Fish Solubles[1,2]

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The presence of "unidentified growth factors" in condensed fish solubles as well as other crude feed supplements has been demonstrated. The nature of these factors was indicated by the work of Camp et al. ('56), Menge et al. ('56), Dannenburg et al. ('55), and Morrison et al. ('55; '56), in which it was observed that the growth response from some crude feed supplements appeared to be due to both inorganic and organic constituents. Several of the purified diets used to study growth factors, however, did not contain adequate amounts of minerals and unsaturated fats. It was indicated by Briggs ('56) that the salt mixtures used by Dannenburg and associates, as well as those used by many other workers, did not meet National Research Council standards. Also, O'Dell and Savage ('57) showed that the basal diet of Dannenburg et al. ('55) was deficient in both potassium and zinc. In addition, O'Dell et al. ('58) referred to the purified diet used by Morrison et al. ('56), in suggesting that it did not contain adequate levels of zinc for normal bone formation and maximum growth.

Bieri et al. ('56) presented data which showed that growing chicks require highly unsaturated fats in their diet for optimal growth. Dam et al. ('58) presented data which indicated that purified diets used by previous workers for the study of unidentified growth factors were low or completely lacking in adequate levels of unsaturated fats. Later, Dam et al. ('59) performed experiments in which potassium, zinc, and corn oil were added to the purified ration at levels necessary for maximal growth. Data accumulated, using this purified ration in growth studies, showed that the growth response from crude supplements was reduced considerably. The results also indicated that the presence of added zinc and potassium eliminated the growth response observed when the ash of the crude supplements was fed. The fact that the growth response was not eliminated completely, however, led Dam et al. ('59) to suggest that an organic factor was present in the crude supplements which aided in the absorption or utilization of the inorganic factors, or both.

The purpose of the present paper is to report the results of attempts to isolate and identify the growth factor(s) in condensed fish solubles.

TABLE 1
Composition of the high-protein basal diet

	%
Soybean oil meal (50% protein)	63.8
Corn (finely ground)	32.0
Dicalcium phosphate	1.4
NaCl (iodized)	0.5
$CaCO_3$	2.0
Methionine	0.1
Vitamin A (20,000 IU/gm)	0.0132
Choline chloride	0.0661
Vitamin E (α-tocopheryl acetate)	0.0150
Vitamin B complex mixture[1]	0.1008
$MnSO_4$ (technical)	0.0176
Vitamin B_{12} mixture[2]	0.0198
Menadione	0.0004
Vitamin D_3 (200,000 ICU/gm)	0.0010

[1] Supplied 2 gm riboflavin, 5 gm calcium pantothenate, 12.5 gm niacin, and 50 gm choline on one pound of ground corn as carrier.
[2] Contained 0.1% vitamin B_{12} on $CaCO_3$ as carrier.

EXPERIMENTAL

During the course of these experiments, 4 different basal rations were used to as-

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say the condensed fish solubles fractions for growth promoting substances. The composition of these basal diets is outlined in tables 1 through 4. Chemical additives to the basal diets were all "technical" or "feed grade" chemicals except for those used in the synthetic diet which were all "reagent grade."

Day-old chicks were weighed, grouped according to weight (a 2-gm spread in each group), and the exceptionally light and heavy chicks discarded. Each group was randomized throughout the experimental pens. Usually, 10 chicks were placed in each pen and 4 replications were used to measure each variant. The chicks were weighed individually at the beginning of each experiment and every week thereafter during the two-week assays and on alternate weeks during the 4-week assays. The differences in mean weight gains were analyzed statistically using the "F" test (Snedecor, '56), and Duncan's ('55) multiple range test. Any statement of probability herein is based on these analyses.

The chicks were maintained in electrically heated batteries equipped with raised, wire floors. The experimental diets

TABLE 2

Composition of regular basal diet

	%
Corn (finely ground)	63.8
Soybean oil meal (50% protein)	32.0
Dicalcium phosphate	1.4
NaCl (iodized)	0.5
$CaCO_3$	2.0
Methionine	0.1
Vitamin A	0.0132
Choline chloride	0.0661
Vitamin E	0.0150
Vitamin B complex mixture[1]	0.1008
$MnSO_4$	0.0176
Vitamin B_{12} mixture[2]	0.0198
Menadione	0.0004
Vitamin D_3	0.0010

[1] Supplied 2 gm riboflavin, 5 gm calcium pantothenate, 12.5 gm niacin, and 50 gm choline on one pound of ground corn as carrier.

[2] Contained 0.1% vitamin B_{12} on $CaCO_3$ as carrier.

TABLE 3

Composition of Diet 303

	%
Finely ground corn	58.5
Soybean oil meal (50% protein)	25.3
Dried whole whey	2.0
DL-Methionine	0.1
Alfalfa meal (17% protein)	2.0
Fish meal (herring)	5.0
$CaCO_3$	1.4
Dicalcium phosphate	2.0
Salt (iodized)	0.5
Yellow grease	3.0
$MnSO_4$	0.018
Vitamin B complex[1]	0.055
Riboflavin	0.003
Vitamin A (20,000 IU/gm)	0.022
Butylated hydroxy toluene (antioxidant)	0.013
Vitamin D_3 (200,000 ICU/gm)	0.005

[1] Supplied 2 gm riboflavin, 5 gm calcium pantothenate, 12.5 gm niacin, and 50 gm choline on one pound of ground corn as carrier.

TABLE 4

Composition of synthetic basal diet

	%
Protein[1]	35.0
Corn oil[2]	16.0
Glycine	1.0
Choline chloride	0.2
Vitamin A	0.050
Vitamin D_3	0.030
Vitamin E	0.020
Ascorbic acid	0.025
Glucose monohydrate[3]	42.000
Methionine (hydroxy analog)	0.600
Butylated hydroxy toluene (antioxidant)	0.020
Mineral mixture(see below)	5.080
Vitamin mixture (see below)	0.020

Mineral mixture

	%
$CaCO_3$	25.0000
K_2HPO_4	15.0000
Na_2HPO_4	12.1600
$Ca_3(PO_4)_2$	23.1800
NaCl	14.6000
$MgSO_4 \cdot 7H_2O$	8.3200
$ZnCO_3$	0.3330
Ferric citrate	0.6600
$MnSO_4 \cdot 4H_2O$	0.7000
KI	0.0870
$CuSO_4 \cdot 5H_2O$	0.0330
NaBr	0.0167
$NaMoO_4 \cdot 2H_2O$	0.0167

Vitamin mixture

	mg/100 lb of diet
Vitamin B_{12} (682 mg of 0.1% vitamin B_{12} on $CaCO_3$ as carrier)	0.682
Biotin	6.800
Menadione	22.700
Pyridoxine	181.500
Folacin	90.800
Riboflavin	363.000
Calcium pantothenate	454.000
Thiamin·HCl	544.500
Niacin	2270.000

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

[2] Mazola, Corn Products Company, New York.

[3] Cerelose, Corn Products Company, New York.

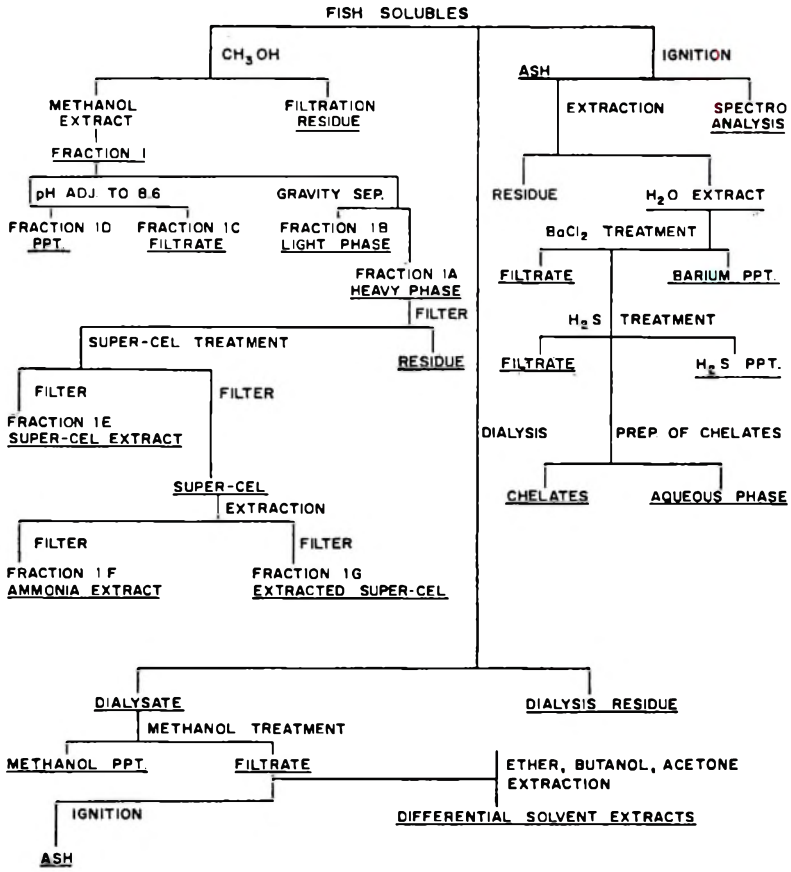

Fig. 1 Schematic flowsheet representing the fractionation of the growth promoting activity of condensed fish solubles.

and tap water were supplied ad libitum throughout the experiments.

"Condensed fish solubles" (sardine origin) obtained from the same commercial lot[4] was used in all experiments. It was added to the basal diets as indicated in the tables. Fractions of the condensed fish solubles added to the diets supplied the activity isolated from the amounts of condensed fish solubles in the positive control diet.

Several techniques were used in attempts to fractionate or concentrate the growth promoting activity of condensed fish solubles. A schematic flowsheet representing these fractionations is presented in figure 1.

One pound of condensed fish solubles was treated with two liters of methanol which had been precooled to 4°C. The protein precipitate which formed was removed by filtration and the methanol removed from the resulting filtrates by vacuum distillation. The remaining aqueous solution is hereafter referred to as fraction 1. Fraction 1 was allowed to stand in a separatory funnel for 7 days in the cold room, during which time it separated into two layers; the aqueous layer was the heavier phase. The two layers were separated, and the aqueous layer and light layer were labeled fractions 1A and 1B, respectively. Another fractionation was performed by adjusting the pH of fraction 1 to 8.6 and allowing a white, crystalline precipitate to form. The precipitate (fraction 1D) was removed by filtration; the

[4] Phillip R. Park Company, San Pedro, California.

filtrate was termed fraction 1C. The dried precipitate, (fraction 1D) lost no weight during ashing at 500°C.

Fraction 1A was diluted with water and Super-Cel was removed by filtration, and the resulting filtrate was labeled 1E. The Super-Cel was resuspended in two liters of 2% ammonia solution and stirred overnight, after which the ammonia extract was filtered and ammonia removed by vacuum distillation. The resulting solution was termed fraction 1F; the Super-Cel remaining after filtration was called fraction 1G.

Several techniques were used to prepare the dialysates. In all cases, commercially available cellophane dialyzer tubing of 1⅞ in. diameter was used. In those cases where the dialysate was collected, 4 loaded tubes were immersed in 7 liters of distilled water which was stirred gently by means of an electric stirrer. Some dialyses were performed at room temperature and others were performed at 4°C; in both cases, the bath was charged anew with distilled water each day for 7 days. The dialysis required a total of 50 liters of water for 4 pounds of condensed fish solubles. Concentration of the dialysates was accomplished by distilling in vacuo.

The use of methanol to precipitate substances at 0°C from the dialysates and the consequential distillation of the methanol from the filtrates was performed essentially in the same manner as that described for fraction 1.

Composite samples of ash of the condensed fish solubles were prepared by heating the liquid material on a hot plate under heat lamps until all moisture was removed and charring occurred. The samples were then placed in the muffle furnace at 650°C and ashed at this temperature for 36 hours. Ashing of the dialysates and dialysis residues was carried out in the same manner. The yield of ash per pound of fish solubles was calculated for several ashings and the average computed was used as the basis for adding ash to the experimental diets at a level equivalent to 12% of condensed fish solubles. The ash averaged 12.0% of the total wet weight of the condensed fish solubles; the individual determinations varied from 11.0 to 12.6%.

Water extracts of the ash were made by treating the ash equivalent of one pound of condensed fish solubles with one liter of boiling distilled water, followed by filtration. Approximately 23% of the ash did not dissolve in the hot water. The hot water extracts were concentrated in vacuo to a volume equal to the weight of the original condensed fish solubles.

Sulfide precipitations were conducted on water extracts of the ash according to the routine quantitative procedures of Griffin and Plunkett ('51). Both acidic and basic sulfide precipitations were made by allowing hydrogen sulfide to bubble through the properly buffered solutions for several minutes. The precipitate was removed immediately by filtration. The filter papers containing the sulfide precipitates were ignited over an open flame until traces of carbon could no longer be distinguished, and the charred residues were ashed in the muffle furnace at 500°C for 24 hours. Finally, the samples were cooled, and ground to a fine powder in a mortar and pestle.

Precipitation of sulfate from the water extracts of the ash was accomplished by using a 10% solution of reagent grade barium chloride.

Analyses of the levels of potassium in the ash of condensed fish solubles were made using the Beckman Model DU spectrophotometer with flame attachment. Flame intensities were read at a wave length of 400.5 mμ with a slit width of 0.09 mm.

Alkaline hydrolysis involved autoclaving a 1:1 dilution of the condensed fish solubles with 6 N sodium hydroxide at 121° for 15 hours, and acid hydrolysis involved the same treatment with 6 N hydrochloric acid. Hydrolysis at its natural pH was performed by simply autoclaving 50% aqueous solution of the condensed fish solubles for 15 hours at 121°C. Autoclaving at pH 7.0 was performed in the same manner after the pH had been adjusted to 7.0 with saturated sodium hydroxide.

RESULTS

Data in this section are presented in as nearly chronological sequence as possible. Levels of statistical significance are based on comparison with the basal diet (tables 1–4) for each experiment unless other-

wise indicated, and are presented as footnotes to the tables. The basal diet used is signified in the body of the tables. Since it was desirable to have a basal diet which would show differences in growth in a short period of time, the "high protein basal" was formulated. The high level of protein in this diet placed the chicks in a state such that they reacted rapidly to dietary changes. It was possible to find growth differences within two weeks by using the "high protein basal," as compared with 4 weeks needed when the "regular basal" was used. Consequently, it was possible to assay large numbers of fractions in considerably less time.

Included in table 5 are observations from early experiments which established the relative stability of the growth promoting substance in condensed fish solubles. It was quite stable to both acid and alkaline hydrolysis.

Fractions 1A through 1D were fed to assay for the growth promoting effect and the results are summarized in table 6. Apparently, the growth promoting activity was soluble in methanol and was not removed from fraction 1 by precipitation at pH 8.6. In experiment 58–6, a good growth response was obtained from this precipitate; however, it was found that a great deal of organic material still remained in the precipitate. When the precipitate was washed carefully with buffer to remove all the organic material, no growth response was obtained from this material (experiment 58–9). It will be noted from inspection of the data that the

TABLE 5

Effect of hydrolysis of fish solubles on growth response

	Diet	Experiment no. 58–10	58–8	59–1
		Average weight gain 1-Week	2-Week	3-Week
		gm	*gm*	*gm*
1	Basal diet (high protein)	36.0	84.2	117.2
2	Basal diet + 2% untreated condensed fish solubles	46.1[2]	104.4[2]	141.9[2]
3	Basal diet + 2% acid hydrolyzed condensed fish solubles	—	100.4[1]	—
4	Basal diet + 2% basic hydrolyzed condensed fish solubles	—	107.9[2]	—
5	Basal diet + 2% condensed fish solubles autoclaved at pH 4.2	—	—	141.8[2]
6	Basal diet + 2% condensed fish solubles autoclaved at pH 2.0	45.8[2]	—	—

[1] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.
[2] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.

TABLE 6

Growth effects of fractions 1A–1D

	Diet	Experiment no. 58–6	58–4	58–9	58–9A	59–1
		2-week average weight gain				
		gm	*gm*	*gm*	*gm*	*gm*
1	Basal diet (high protein)	152.8	127.8	125.3	76.9	117.2
2	Basal diet + 2% condensed fish solubles	170.6[1]	146.2[2]	140.8[1]	103.2[2]	141.9[2]
3	Basal diet + fraction 1[3]	—	139.8[1]	132.4	—	—
4	Basal diet + fraction 1C[3]	176.1[1]	—	132.8	92.5[1]	136.5[2]
5	Basal diet + fraction 1D[3]	173.8[1]	—	110.8	—	—
6	Basal diet + fraction 1B[3]	—	—	—	86.4	121.0
7	Basal diet + fraction 1A[3]	—	—	—	98.5[2]	—

[1] Differences between mean and the mean growth on basal ration significant at $P < 0.05$.
[2] Differences between mean and the mean growth on basal ration significant at $P < 0.01$.
[3] Fractions supplied the amount of activity isolated from 2% of condensed fish solubles.

light layer (fraction 1B) did not have appreciable growth promoting activity, whereas the heavy layer (fraction 1A) contained appreciable growth promoting activity.

Fractionations designed to concentrate further the growth promoting activity noted in fraction 1, by adsorption and elution techniques, were unsuccessful as indicated by the data summarized in table 7. It was obvious that some adsorption of the active substances occurred on Super-Cel, but in all cases some active material remained unadsorbed. Indeed, it was not possible to determine which fraction contained the greater part of the growth promoting activity. Consequently, this technique was abandoned in favor of dialysis.

Several experiments were performed in an attempt to use dialysis as a means of initial separation of the growth promoting substances. It was also hoped that dialysis might help establish whether one or more components were contributing to the growth promoting effect. The possibility of the existence of a nondialyzable component was eliminated by exhaustively dialyzing condensed fish solubles against running tap water and then checking the residue (nondialyzable material) for a growth response. When this was done, no growth promoting effect was found in the residue. Dialysis in the cold room (4°C) was so slow that complete removal of the growth activity was not affected after repeated attempts. The methanol-insoluble material in the dialysate was devoid of any of the growth promoting substance, whereas the methanol-soluble material was quite active. The amount of precipitate

TABLE 7

Growth effects of fractions 1E, 1F, and 1G

	Diet	Experiment no. 58–5	58–6	58–9A
		2-week average weight gain		
		gm	*gm*	*gm*
1	Basal diet (high protein)	136.0	152.8	76.9
2	Basal diet + 2% condensed fish solubles	160.0[2]	170.6[1]	103.2[2]
3	Basal diet + fraction 1E[3]	152.8[2]	167.0[1]	108.0[2]
4	Basal diet + fraction 1F[3]	154.0[2]	164.4[4]	100.4[2]
5	Basal diet + fraction 1G[3]	157.5[2]	164.0[4]	89.8

[1] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.
[2] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.
[3] Fractions supplied the amount of activity isolated from 2% of condensed fish solubles.
[4] Approaches significance at $P < 0.05$.

TABLE 8

Summary of dialysis experiments

	Diet	Experiment no. 59–10	59–13
		2-week average weight gain	
		gm	*gm*
1	Basal diet (high protein)	113.3	130.1
2	Basal diet + 2% condensed fish solubles	141.6[1]	158.0[2]
3	Basal diet + dialysis of condensed fish solubles (at room temperature)[3]	137.7[1]	155.8[2]
4	Basal diet + dialysis residue[3]	—	135.3
5	Basal diet + dialysis residue[3] (against tap water)	114.3	—
6	Basal diet + methanol precipitate of dialysate[3]	—	131.7
7	Basal diet + methanol-soluble material of dialysate[3]	—	145.7[1]
8	Basal diet + ash of dialysate[3]	136.0[1]	—
9	Basal diet + ash of condensed fish solubles[3]	129.8	—

[1] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.
[2] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.
[3] Fractions supplied the amount of activity isolated from 2% of condensed fish solubles.

TABLE 9

Effect of ash of fish solubles on growth

	Diet	Experiment no.	
		59–10	58–94
		2-week average weight gain	
		gm	*gm*
1	Basal diet (high protein)	113.3	76.9
2	Basal diet + condensed fish solubles	141.6[1]	103.2[2]
3	Basal diet + ash of fish solubles[3]	129.8[3]	99.1[2]

[1] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.
[2] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.
[3] Fractions supplied the amount of material obtained from 2% of condensed fish solubles.

was relatively small, since most of the methanol-insoluble material was retained on the cellophane membrane during dialysis. Attempts to separate further by phase distribution in such solvents as *n*-butanol, acetone, and ether, failed because no measurable amount of the methanol-soluble material was extracted by these solvents. These observations are summarized in table 8.

At the same time, experiments were being carried out in an attempt to effect a concentration of the growth promoting substance by treatment of the ash of condensed fish solubles in various ways. This work was prompted by the results obtained by Morrison et al. ('55; '56) and Camp et al. ('55), while working with the ash of condensed fish solubles and mixtures of growth promoting substances. Also, in view of the results obtained in experiments reported here involving the dialysate and the ash of the dialysate, it was felt that fractionation of the ash of condensed fish solubles demanded special attention.

First, it was necessary to ascertain whether ash of the condensed fish solubles was indeed active in promoting a growth response and whether the magnitude of the response was of the same order as that obtained from the intact condensed fish solubles. The results of the experiments designed to provide these data are summarized in table 9.

All treatments produced significant increases in growth over that obtained with the basal diet with the exception of the treatment containing the ash of condensed fish solubles in experiment 59–10. However, the difference here was great enough to approach significance at the 0.05 level of probability. It was noted in other experiments, the results of which are reported herein, that all diets containing condensed fish solubles or the ash of condensed fish solubles consistently gave a growth response even though the differences were not always large enough to be significant. The most logical step in the initial fractionation of the ash seemed to be a water extraction and subsequent filtration.

The ash was not included in experiments 59–1 and 59–14; hence it was impossible to make comparisons between the water extract and the untreated ash (table 10). But it was obvious from these experiments that the ash contained a growth promoting substance and that the water extraction was effective in removing it completely.

When the same treatments were applied using the regular basal diet, the difference in responses between condensed fish solubles and a water extract of the ash seemed to disappear. This is indicated by the data in table 11. The disappearance of this difference was noted in several experiments conducted during this study. These experiments also showed that the magnitude of the growth response was decreased when the regular basal diet was used. In fact, the difference in growth was not always significant when condensed fish solubles were added at the 2% level to the regular levels. When condensed fish solubles were added at the 4% level, a significant growth response nearly always occurred. Also, the difference in growth response between the ash and condensed fish solubles reappeared when the ash and condensed fish solubles were fed at the 4% level (experiment 60–2).

In view of the relatively large amount of sulfate in condensed fish solubles, an

TABLE 10

Growth activity of aqueous extracts of ash of fish solubles

	Diet	Experiment no. 58–11	59–1	59–14
		2-week average weight gain		
		gm	*gm*	*gm*
1	Basal diet (high protein)	94.9	117.2	106.4
2	Basal diet + 2% condensed fish solubles	110.6[1]	141.9[2]	135.5[2]
3	Basal diet + ash of fish solubles[3]	110.5[1]	—	—
4	Basal diet + water extract of ash[3]	102.8[4]	132.5[2]	116.3
5	Basal diet + residue from water extract[3]	97.7	117.3	107.5

[1] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.
[2] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.
[3] Fractions supplied the amount of material obtained from 2% of condensed fish solubles.
[4] Approaches significance at $P < 0.05$.

TABLE 11

Growth activity of ash of fish solubles

	Diet	Experiment no. 59–15	60–2
		4-week average weight gain	
		gm	*gm*
1	Basal diet (regular)	401.0	373.8
2	Basal diet + 2% condensed fish solubles	428.7[1]	—
3	Basal diet + 4% condensed fish solubles	—	429.0[1]
4	Basal diet + water extract of ash	427.9[1,2]	396.8[3]

[1] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.
[2] Contained the amount of material isolated from 2% of condensed fish solubles.
[3] Contained the amount of material isolated from 4% of condensed fish solubles.

experiment was designed to determine whether sulfate was causing the response. From the results of this experiment, it was concluded that sulfate was not involved in the growth response. In an attempt to determine whether a specific group of cations was involved in producing the growth response, several experiments were conducted involving hydrogen sulfide treatment of water extracts of the ash. It was found that the growth effect of the water extract of ash was either not affected by treatment with hydrogen sulfide, or if a decrease in growth response occurred, it was very slight. These results were interpreted to mean that either the growth promoting substance was not cationic, or its sulfide was soluble under the conditions of these experiments. The latter possibility seemed very likely if the growth promoting substance(s) was cationic but present in concentrations of the order of 1 ppm or less. Hydrogen sulfide precipitations were performed in both acidic and alkaline media and in the presence and absence of phosphate.

It was also deemed desirable to determine whether the factor(s) exerted itself when fed in a purified-type diet; thus the synthetic basal diet (table 4) was used. The results obtained with this diet are summarized in table 12. In these experiments the condensed fish solubles did not elicit a significant response. Previous feeding trials (not reported herein) had shown the synthetic diet to be capable of supporting growth equal and possibly superior to the other diets used; thus it was difficult to explain this difference in response.

Because a large amount of the activity was apparently in the ash, the mineral constituents of the two diets were studied. Several trace elements normally considered to be present in natural diets had not been added to any of the diets except the synthetic diet. A review of analytical data available allowed the elimination of all except copper, zinc, and molybdenum. There-

TABLE 12

Growth effect of synthetic basal diet with added fish solubles

	Diet	Experiment no. 59–1	59–10	59–20	59–21	60–5
		Average weight gain				
		14-Day	11-Day	18-Day	26-Day	30-Day
		gm	*gm*	*gm*	*gm*	*gm*
1	Basal diet (synthetic)	170.2	122.7	238.2	327.0	610.0
2	Basal diet + 2% condensed fish solubles	181.3	120.3	—	339.0	619.0
3	Basal diet + 4% condensed fish solubles	—	—	—	—	619.0
4	Basal diet + methanol extract of fish solubles	174.5[1]	—	—	—	—
5	Basal diet + ash of condensed fish solubles	—	112.8[1]	231.9[1]	—	—

[1] Contained the amount of material isolated from 2% of condensed fish solubles.

TABLE 13

Growth promoting effect of added Cu, Zn, and Mo[1]

	Diet	Experiment no. 59–14
		2-week average weight gain
		gm
1	Basal diet (high protein)	106.4
2	Basal diet + 2% condensed fish solubles	135.5[3]
3	Basal diet + water extract of ash[2]	116.7
4	Basal diet + Cu + Mo	116.1
5	Basal diet + Cu + Mo + H_2O extract of ash[2]	115.7
6	Basal diet + Zn + Mo	106.8
7	Basal diet + Zn + Mo + H_2O extract of ash[2]	115.8
8	Basal diet + Cu + Zn	112.5
9	Basal diet + Cu + Zn + H_2O extract of ash[2]	117.3
10	Basal diet + Cu + Zn + Mo	102.2
11	Basal diet + Cu + Zn + H_2O extract of ash[2]	117.7

[1] Cu added as $CuSO_4 \cdot 5H_2O$, Zn as $ZnSO_4$, Mo as $Na_2MoO_4 \cdot 2H_2O$, at levels added to the synthetic diet (table 4).

[2] The amount of material isolated from 2% of condensed fish solubles.

[3] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.

fore, an experiment was designed to test the growth promoting effect of these three elements individually and in combination. The levels of reagent grade salts added to the basal diet were equal to those added to the synthetic diet. The results of this experiment are summarized in table 13. An inspection of the data included in table 13 indicated that copper and molybdenum might be responsible for the growth response.

Several experiments were conducted to determine whether copper alone could have been the growth promoting substance. The results of these experiments are summarized in table 14. From an inspection of the data included in this table, it was evident that a serious contradiction had arisen. Experiments 59–19 and 59-21 clearly indicated that copper alone was not the growth promoting substance. Yet, in experiment 59–15, highly significant growth responses were obtained from low-level additions of copper. It appeared that under certain conditions, copper was able to promote growth.

In order to attack the problem from another direction, a complete spectrographic analysis of the ash of the condensed fish solubles was obtained.[5] A tabulation of elements present in the ash is included in

[5] Spectrographic analysis supplied through courtesy of Charles Pfizer and Company, Terre Haute, Indiana.

TABLE 14

Growth promoting effect of added Cu

	Diet	Experiment no. 59–15 Average weight gain 4-Week	59–19 3-Week	59–21 3-Week
		gm	*gm*	*gm*
1	Basal diet (regular)	401.2	238.1	—
2	Basal diet (regular) + 2% condensed fish solubles	428.7[1]	257.0	—
3	Basal diet (regular) + water extract of ash[3]	427.9[1]	251.8	—
4	Basal diet (regular) + 9 ppm Cu	393.1	232.4	—
5	Basal diet (regular) + 18 ppm Cu	434.6[2]	226.2	—
6	Basal diet (regular) + 36 ppm Cu	430.5[2]	226.3	—
7	Basal diet (regular) + 18 ppm Cu + water extract[3] of ash	426.3[1]	—	—
8	Basal diet (regular) + 18 ppm Cu + 2% condensed fish solubles[3]	—	254.3	—
9	Basal diet (synthetic)	—	—	238.0
10	Basal diet (synthetic) + ash of condensed fish solubles[3]	—	—	232.0
11	Basal diet (synthetic) + added Cu	—	—	233.0
12	Basal diet (synthetic) + ash of condensed fish solubles[3,4] + added copper	—	—	236.0

[1] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.
[2] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.
[3] The extracts contained the amount of activity isolated from 2% of condensed fish solubles.
[4] Cu added as $CuSO_4 \cdot 5H_2O$ at levels added to synthetic diet (table 4).

table 15. These data indicate that aluminum and chromium are also present in appreciable amounts.

A complete factorial experiment (data from this experiment not given in tabular form) was designed to determine whether copper, zinc, aluminum and chromium, either alone or in combination, were responsible for the inorganic growth response. The elements were added to the diets in the form of reagent grade salts. No growth response was observed either with the elements alone or in combination when they were added at levels found in 4% condensed fish solubles. Significant interaction occurred only with condensed fish solubles. Having eliminated the possibility of these 4 elements causing the growth response, experiments were conducted in which copper and molybdenum were added at the levels observed in condensed fish solubles. The results of these experiments are tabulated in table 16. The copper and molybdenum combination resulted in a highly significant growth response in experiment 60–1, and was not significantly different from that of the water extract of the ash. In experiments 60–2 and 60–5, there was no significant response from the combination of the two. Even so, it will be noted that the combination of copper and molybdenum elicited a response which was 40% of the potential growth increase as measured by the positive control. In both experiments 60–2 and 60–5, the response from condensed fish solubles was also very small.

It has been shown that supplementation of the synthetic diet with condensed fish solubles, or fractions thereof, failed to give a growth response. Consequently, it was decided that the growth rate of chicks

TABLE 15

Analysis of ash of condensed fish solubles

	gm/100 gm ash
Mg	2.500
Ca	1.000
Si	0.500
Fe	2.000
Al	0.100
Cu	0.200
Mn	0.020
Cr	0.100
Co	0.001
Ni	0.070
Pb	0.010
Zn	0.900
Mo[1]	0.005

[1] Determined by method of Purvis ('56).

TABLE 16

Effects of Mo and Cu on growth[1]

	Diet	Experiment no. 60–1	Experiment no. 60–2	Experiment no. 60–5
		4-week average weight gain		
		gm	*gm*	*gm*
1	Basal diet (regular)	378.7	373.9	417.9
2	Basal diet + 4% condensed fish solubles	464.0[3]	429.0[2]	454.8[2]
3	Basal diet + water extract of the ash[4]	426.0[3]	—	—
4	Basal diet + Mo + Cu	412.2[3]	398.7	432.7
5	Basal diet + Mo	—	394.7	415.2
6	Basal diet + Cu	—	398.0	398.7

[1] Mo added as $Na_2MoO_4 \cdot 2H_2O$ and Cu as $CuSO_4 \cdot 5H_2O$ at levels found in condensed fish solubles.

[2] Differences between mean and the mean growth with basal ration significant at $P < 0.05$.

[3] Differences between mean and the mean growth with basal ration significant at $P < 0.01$.

[4] Contained the activity isolated from 4% of condensed fish solubles.

TABLE 17

Comparative effects of synthetic and 303 basal diets on growth

	Diet	Experiment no. 59–21
		26-day av. weight gain
		gm
1	Basal diet (303)	328.7
2	Basal diet (303) + 2% condensed fish solubles	328.4
3	Basal diet (synthetic)	326.8
4	Basal diet (synthetic) + 2% condensed fish solubles	359.0

fed the synthetic diet should be compared with that of the chicks receiving the 303 basal diet (table 3). The results tabulated in table 17 indicated very strongly that the synthetic diet was supplying the need for the unidentified growth factors which were included in diet 303 (a commercial-type diet containing 5% of fish meal). Consequently, the conclusion might be drawn that there was no unknown organic growth factor present in condensed fish solubles. In previous experiments, however, results were obtained with the regular and high-protein basal diets which suggested a growth response in addition to that supplied by the inorganic constituents alone. An explanation of the apparent contradiction was the possibility of a synergism in which the organic response was due to the ability of some organic compounds to aid in the absorption and utilization of cations causing the inorganic response by chelation or similar processes. The existence of this type of synergism seems even more plausible when one considers recent reports of the increased utilization of certain cations chelated with ethylenediaminetetraacetic acid (Kratzer et al., '59). The presence of a chelating agent in the synthetic diet that would account for the absence of a growth stimulation in the presence of condensed fish solubles was not immediately obvious. It was thought, however, that the isolated soybean protein might be acting in the capacity of a chelating agent. Analysis of this soy protein[6] by the manufacturer showed that considerable quantities of cations including zinc, copper, aluminum, and others are associated with this protein. O'Dell found a considerable amount of molybdenum associated with this soybean protein and that the molybdenum was nearly completely available to the chick.[7] The ability of this protein to bind these cations so tenaciously that they were not lost during the acidic precipitation of the protein indicates binding which is quite stable under acidic conditions such as that in chelates. But it is interesting to note that both Dam and Morrison washed the soy protein before incorporating it into their synthetic diets. The removal of ions during washing could account for the growth response these workers observed when the synthetic diet was supplemented with a mixture of

[6] ADM C-1 Assay Protein, Archer-Daniel-Midland Company, Cincinnati.

[7] Personal communication with B. L. O'Dell.

sources of the growth factors. The soy protein used in the synthetic basal in the work reported in this paper was not washed, and it was felt unnecessary in view of the growth rate of birds receiving the synthetic basal.

SUMMARY

Both inorganic and organic compounds were involved in the growth response that was observed when chicks were fed diets containing condensed fish solubles. It appeared that the total growth response observed was due to both an inorganic and organic compound(s). The necessary inorganic constituents included copper and molybdenum.

The growth promoting substance was extremely stable and completely dialyzable across a cellophane membrane. Considerable concentration of the factor was affected by dialysis followed by precipitation with methanol.

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Effect of Fasting on Serum and Liver Lipid Levels in the Rat[1]

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In dietary studies of lipid metabolism, the procedure and degree of fasting the rats, prior to the removal of blood and tissue samples, may affect the results obtained on the samples as well as the statistical interpretation of the results. Kohn ('50) studied changes in the plasma of the rat during fasting and reported that the plasma cholesterol levels were not affected by fasting. Similar results have been reported by Sure et al. ('33). More recent work by Ridout and associates ('54) has emphasized the importance of the time interval between the last meal and the collection of the blood sample when the diet contains lipotropic agents and excessive amounts of cholesterol. They report that the postprandial elevation in bound serum cholesterol was related to the concentration of cholesterol in the ration.

Various workers have used different periods of fasting in their studies of lipid metabolism. Okey and Lyman ('57) removed food cups from their cholesterol-fed rats at 10 PM the evening preceding the morning of autopsy; Passananti et al. ('58) state that care was taken to insure a uniform time interval between the removal of food from the cage and the securing of tissues from the rat; Nath and co-workers ('59) withdrew blood from the rat after a 15- to 20-hour fasting period; Vahoundy et al. ('59) sacrificed their rats while in the postabsorptive state; Harrill et al. ('59) fasted their rats for 4 hours prior to decapitation and withheld the riboflavin supplement for 48 hours, whereas Coleman and Beveridge ('60) removed food cups 7 hours before bleeding to ensure a fasting period of at least that duration.

Attention has also been given to the effect of fasting on cholesterol synthesis by the liver. The work of Lyon and associates ('52) and Tomkins and Chaikoff ('52) showed that fasting the rat for 24 hours or longer reduced the liver's ability to incorporate acetate carbon into cholesterol; and although this is not relevant to the present report it shows the extensive influence of fasting on some of the major body tissues such as the liver.

Recent work conducted in this laboratory concerned the effect of feeding diets containing two types of fat on serum and liver cholesterol levels in rats. Half of the experimental groups of rats were fasted prior to sacrifice and half were not restricted in access to their food. The resulting data indicated not only decided differences in tissue lipid levels between the fasted and nonfasted rats but certain statistical significances were evident in the nonfasted rats that were not noted in the fasted groups. The work to be reported here describes this portion of the study and these differences.

METHODS

Weanling rats of the Holtzman strain[2] were assigned at random and with a balanced sex ratio to the experimental groups. They were placed in individual cages and fed a commercial laboratory chow[3] diet ad libitum for 24 days at which time the female rats weighed an average of 145 gm and male rats 186 gm. They were then fed, ad libitum, one of the experimental diets described in table 1 and food consumption and rat weights were recorded

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[2] Obtained from Holtzman Rat Company, Madison, Wisconsin.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 1

Composition of diets[1]

Ingredients[1]	Groups			
	1, 2	3, 4	5, 6	7, 8
	%	%	%	%
Composition				
Nonfat dry milk solids	62.5	61.4	58.9	57.8
Corn oil	25.9	25.4	—	—
Margarine	9.4	9.2	—	—
Butter	—	—	38.9	38.2
Mineral mix	—	1.8	—	1.8
Vitamin mix	2.2	2.2	2.2	2.2
Characteristics				
Cal. from fat, %	56.5	56.5	56.4	56.4
Cal. from protein, %	16.6	16.7	16.7	16.7
Cal./10 gm diet	53.5	52.4	50.3	49.4
Cal./gm protein	24.1	24.2	24.0	24.0

[1] Description of ingredients: nonfat dry milk solids, Pet and Carnation Brands; corn oil, Mazola; margarine, Nucoa; butter, Gallatin Maid; mineral mixture, Salt Mixture USP XIV, Nutritional Biochemicals Corp., Cleveland; vitamin mix, Vitamin Diet Fortification Mixture (dextrose base), Nutritional Biochemicals Corp., Cleveland, used so that 100 gm diet contained the following in mg: vitamin A concentrate, 9.9; vitamin D concentrate, 0.55; α-tocopherol, 11.0; ascorbic acid, 99.0; inositol, 11.0; choline chloride, 165; menadione, 5.0; *p*-aminobenzoic acid, 11.0; niacin, 9.9; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; and the following in μg per 100 gm diet: biotin, 44; folic acid, 198; and vitamin B_{12}, 3.0.

during a 10-week feeding period. Iodine numbers of the fats used were: corn oil, 122.8; margarine, 81.5; and butter, 33.7. Characteristics of the diets such as percentage of calories from fat and from protein, calories per gram of protein and calories per 10 gm of diet are also shown in table 1 since the dietary ingredients were used in amounts so that the first three factors remained fairly constant among diets.

Rats in groups 1, 3, 5, and 7, had access to their food up to the time of decapitation; those in groups 2, 4, 6, and 8, had their food cups removed approximately 10 to 12 hours prior to sacrifice. At the end of the experimental period, rats were decapitated, blood was collected for serum preparation and livers were removed, weighed and packaged in polyethylene wrap. Serum samples and livers were frozen and held at − 23°C until analyzed.

Total cholesterol in the serum samples was determined by the method of Abell et al. ('52). Serum lipid phosphorus determinations were made following the hydrazine sulfate method of Boltz and Mellon ('47) and as modified by Beveridge and Johnson ('49). An homogenate was prepared from 1.5 gm of liver and 35 ml of alcohol-ether mixture (Bloor, '28). The mixture was transferred quantitatively with washings to a 100-ml volumetric flask, brought to a boil on a water bath, cooled, made to volume with the alcohol-ether mixture and filtered. Aliquots from this filtrate were used for determining total liver lipids and total cholesterol. Total lipids were determined following the method of Bloor ('28) and total cholesterol using the method of Abell et al. ('52).

RESULTS AND DISCUSSION

Serum and liver lipid levels in the fasted and nonfasted rats are shown in table 2. Not shown in the table is the following information on growth responses to the various diets during the 10-week experimental period. Female rats in the nonfasted groups 1, 3, 5, and 7, gained an average of 56, 67, 61, and 75 gm, respectively; males in the same groups gained 116, 191, 105, and 187 gm. In the fasted groups 2, 4, 6, and 8, females gained an average of 89, 79, 84, and 87 gm, whereas the males in the same groups gained 173, 222, 167, and 202 gm, respectively. A direct comparison of the various lipid levels between rats so treated (table 2) indicates that all nonfasted rats had higher serum cholesterol levels, larger phospholipid cholesterol ratios, heavier liver weights and lower liver lipid and liver cholesterol levels than did the fasted rats receiving similar diets.

However, when the data concerning liver lipids (percentage of moist weight and milligrams per liver), liver cholesterols (milligrams per gram and milligrams per liver) and serum cholesterols were subjected to an analysis of variance[4] certain differences were significant in the nonfasted rats that were not apparent in the fasted rats. These differences will be noted and discussed.

[4] Calculations were made by the Statistical Laboratory, Montana State College.

TABLE 2

Liver and serum lipids of fasted and nonfasted rats

Group no. and diet description	Fast[1]	No. of rats	Liver values					Serum values	
			Weight	Total lipids		Total cholesterol		Phospholipid cholesterol ratio	Total cholesterol
			gm	*% moist weight*	*mg/liver*	*mg/gm*	*mg/liver*		*mg/100 ml*
Females									
1 Vegetable fat	none	12	7.5 ± 0.28[2]	4.7 ± 0.13	351	2.6 ± 0.15	20	3.2	126.3 ± 11.1
2 Vegetable fat	yes	5	6.5 ± 0.21	5.8 ± 0.32	377	3.2 ± 0.16	21	2.2	89.4 ± 4.3
3 Vegetable fat and mineral	none	6	7.4 ± 0.20	4.6 ± 0.16	340	2.7 ± 0.12	20	3.6	127.5 ± 8.9
4 Vegetable fat and mineral	yes	5	6.1 ± 0.35	4.8 ± 0.14	289	3.2 ± 0.11	19	1.8	87.1 ± 2.5
5 Animal fat	none	12	7.4 ± 0.33	5.3 ± 0.24	386	2.5 ± 0.25	19	3.3	132.0 ± 4.3
6 Animal fat	yes	11	6.6 ± 0.10	6.1 ± 0.23	400	3.1 ± 0.09	20	2.0	104.8 ± 5.9
7 Animal fat and mineral	none	6	7.9 ± 0.45	4.7 ± 0.27	366	2.6 ± 0.13	21	3.8	119.4 ± 5.8
8 Animal fat and mineral	yes	6	6.2 ± 0.05	5.7 ± 0.39	352	3.1 ± 0.14	19	2.0	85.8 ± 3.5
Males									
1 Vegetable fat	none	12	11.3 ± 0.50	4.8 ± 0.11	540	3.0 ± 0.08	33	3.1	109.7 ± 5.3
2 Vegetable fat	yes	5	10.6 ± 0.51	5.2 ± 0.27	543	3.2 ± 0.16	34	2.0	81.3 ± 2.6
3 Vegetable fat and mineral	none	6	13.5 ± 0.66	5.2 ± 0.23	703	3.0 ± 0.13	41	3.6	107.3 ± 2.9
4 Vegetable fat and mineral	yes	5	11.7 ± 0.38	5.1 ± 0.13	594	3.4 ± 0.17	39	1.9	76.7 ± 2.4
5 Animal fat	none	12	11.1 ± 0.44	4.6 ± 0.17	517	2.6 ± 0.07	29	2.9	120.5 ± 4.8
6 Animal fat	yes	11	9.6 ± 0.30	4.8 ± 0.11	449	3.1 ± 0.14	29	1.9	71.9 ± 2.2
7 Animal fat and mineral	none	6	13.7 ± 0.47	5.3 ± 0.20	733	3.8 ± 0.26	52	3.6	104.7 ± 4.2
8 Animal fat and mineral	yes	6	10.2 ± 0.41	5.1 ± 0.31	523	3.0 ± 0.14	31	1.9	74.6 ± 4.1

[1] Fasted rats had food cups removed 10 to 12 hours prior to sacrifice.

[2] Standard error of the mean.

Liver weights of the fasted rats were markedly lower than those of the nonfasted rats. Okey and co-workers ('60) reported similar differences in liver weights upon overnight fasting. They suggested that water and glycogen may account for most of the weight lost in overnight fasting and that the percentage concentration of lipid is therefore a poor index of liver lipid retention.

Although differences in total lipids in the liver, percentage of moist weight and milligrams per liver, due to fasting are statistically significant ($P < 0.01$), there is also interaction between the groups. The tendency for fasted rats to have higher total lipids, percentage of moist weight, than nonfasted rats, though statistically significant, is noted only in 6 of the 8 groups. However, due to the loss in weight of the livers upon fasting, the reverse was true for liver lipids expressed on a milligram per liver basis; 5 of the 8 groups had higher lipid values when the rats were not fasted. The difference in total lipids, milligrams per liver, due to type of fat was significant ($P < 0.05$) when rats were not fasted, but was not significant when the rats were fasted.

Male rats, not fasted prior to sacrifice, had higher liver cholesterol values, expressed as milligrams per gram or milligrams per liver, than did the females. When the rats were fasted, liver cholesterol values on a milligrams per gram basis were similar for males and females; but when liver weights were taken into consideration, and the values were expressed as milligrams per liver, male rats had higher cholesterol values than did the females. In most instances, when liver cholesterol values were expressed on a milligrams per liver basis, fasted and nonfasted rats were quite similar, due to the change in the weight of the liver brought about by fasting.

Serum cholesterol levels of nonfasted rats were significantly higher ($P < 0.01$) when the rats were fed the animal-fat-containing diets than when they received diets containing the vegetable fat. These differences were no longer significant when the rats were fasted prior to decapitation. The diets made with the animal fat contained an estimated 0.1% of cholesterol supplied by the butter; those with the vegetable fat contained no cholesterol. Although neither of the diets contained any appreciable amount of cholesterol, fasting the rat prior to sacrifice lowered the serum cholesterol to such a degree that differences brought about by the ingestion of the two types of fat were no longer evident. The groups of rats not fasted prior to sacrifice were treated in an otherwise identical manner to those that were fasted. The significant differences in the serum cholesterol levels of these nonfasted rats, due to the type of fat fed, are considered to be of physiological significance and the higher serum cholesterol level observed in the rats fed the animal-fat-containing diet represents a true, influencing condition of the circulating blood stream of the rat for the major portion of the normal 24-hour day. Ridout et al. ('54) have reported that elevated serum cholesterol values, related to the concentration of cholesterol in the diet, returned slowly to normal after about 18 to 24 hours. Interpretation of the data presented in table 2 indicates that, under certain dietary and experimental conditions such as those used in this study, serum cholesterol levels may also be significantly elevated when the diets contain no appreciable amount of cholesterol. Furthermore, if this elevated serum cholesterol does not return to normal until 18 to 24 hours after the ingestion of food, cholesterol values of serum from nonfasted rats would be more representative of the condition of the blood stream for the major portion of the 24-hour day than would that from fasted rats.

Although the phospholipid-cholesterol ratios were not included in the statistical analysis of the data, they are presented in the table. All ratios for nonfasted rats were higher than those from the fasted rats.

SUMMARY

Under the experimental conditions of this study, the procedure of fasting rats 10 to 12 hours prior to obtaining liver and serum samples for lipid determinations affected the amount of lipid and cholesterol present in the tissues and serums. This procedure also affected the statistical interpretation of the results.

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Interrelationships Between Magnesium and Fluoride in Chicks[1,2]

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Warburg and Christian ('42) observed that the quantity of fluoride necessary for one-half inhibition of enolase was reduced by increasing the magnesium concentration of the media. It was also found that when the phosphate buffer was replaced with bicarbonate-carbonic acid buffer, no inhibition of enolase by fluoride could be demonstrated. The authors postulated the formation of a magnesium fluorophosphate-enzyme complex which renders the enolase inactive.

A similar relationship between magnesium and fluoride was afforded by the *in vitro* studies on calcification by Goldenberg and Sobel ('51). Calcification was inhibited by fluoride in the presence of magnesium, but not in its absence.

In vivo studies on fluoride and magnesium interrelationships are limited, and one report stated that magnesium had no effect on fluoride toxicity in rats (Schuck, '38), whereas another suggested that magnesium inhibited a fluoride toxicity (Dibak and Ginter, '59).

In view of the demonstrated relationship between magnesium and fluoride on enzymatic reactions *in vitro*, it was deemed advisable to study further *in vivo* the interrelationship between magnesium and fluoride, and also to ascertain the effect of phosphorus level in the diet on this relationship.

EXPERIMENTAL

Male, crossbred chicks (Vantress male × Hubbard female) were used in both experiments. The chicks were placed on treatment at one day of age in electrically heated, wire-floor batteries with feed and water supplied ad libitum. Individual weights were recorded weekly over the 4-week experimental period.

The composition of the basal diet in percentage of the total ration was as follows: ground yellow corn, 46.9; dehulled soybean oil meal, 36.0; condensed fish solubles, 4.0; soybean oil, 5.0; vitamin premix, 1.1; sodium chloride (iodized), 0.45; manganese sulfate (technical grade), 0.05; DL-methionine, 0.15. Dicalcium phosphate was added at a level of 1.50 or 2.95% to give 0.70 or 1.00% of phosphorus, respectively, in the diet. Limestone was added to the diets containing 1.50 or 2.95% of dicalcium phosphate at a level of 1.45 or 0.45%, respectively, so that the calcium level was maintained at 1.0% in all diets. A fluoride level of 0.08% was obtained by the addition of reagent grade sodium fluoride to the specified diets. Reagent grade magnesium carbonate was added to the specified diets at a level of 1.0% which supplied 0.25% of magnesium. Glucose[4] was used to adjust the rations to 100%.

The vitamin premix supplied the following in amounts per 100 gm of diet: vitamin A, 1000 IU; vitamin D_3, 150 ICU; vitamin E acetate, 0.56 IU; choline chloride, 150 mg; niacin, 3.17 mg; D-calcium pantothenate, 1.41 mg; riboflavin, 0.71 mg; menadione sodium bisulfite, 0.07 mg; procaine penicillin, 0.44 mg; vitamin B_{12}, 0.66 μg.

Experiment 1 was factorial in design involving two levels of supplemental fluoride (none and 0.08%) and two levels of supplemental magnesium (none and 0.25%). Experiment 2 was also factorial

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[4] Cerelose, Corn Products Company, New York.

in nature with the same levels of fluoride and magnesium as in experiment 1, duplicated on two levels of phosphorus (0.7 and 1.0%). The experimental diets were fed to three replicates of 15 chicks each in experiment 1, and three replicates of 10 chicks each in experiment 2.

At the end of the 4-week experimental period, blood samples were taken from birds in each treatment by cardiac puncture with heparin as an anticoagulant. The plasma proteins were precipitated with trichloroacetic acid (10%) and a clear supernatant obtained by centrifugation. Aliquots of the clear supernatant were used to determine plasma magnesium by the method of Young and Gill ('51) and plasma inorganic phosphorus by the method of Fiske and Subbarow ('25).

After obtaining the blood samples in experiment 2, the birds were killed and the right femur removed for bone analyses. Individual analyses were run on each of the 5 femurs within a treatment. Femurs were extracted for 16 hours with absolute ethyl alcohol followed by 16-hour extraction with ethyl ether in a Soxhlet extractor. The bones were dried in a vacuum oven and ashed in a muffle furnace at 600°C for 18 hours. The ash was calculated as a percentage of the dry, fat-free bone weight. Calcium and phosphorus content of the bone was determined by the oxalate and molybdate methods as outlined in the Official Methods of Analyses (AOAC, '60).

Statistical analysis of the data was made by the analysis of variance (Snedecor, '56). In the design of both experiments, chicks were assigned at random to the various replicates and treatments; however, the replicates were assigned so that no replicate of a treatment could be in the same deck level or the same battery. Thus, the replicates were in actuality deck levels, and therefore, replicates were considered fixed in analyzing the data. This applies only to the analysis of 4-week weights since replicates were not considered in evaluating the chemical analyses of blood and bone.

RESULTS AND DISCUSSION

Experiment 1. The results of this experiment are presented in table 1. Statistical analysis of the 4-week weights revealed a highly significant ($P < 0.01$) depression in growth as a result of added dietary fluoride. A significant reduction in growth ($P < 0.05$) was found due to supplemental magnesium, but a significant ($P < 0.05$) magnesium × fluoride interaction was also observed. A comparison of the means would suggest that the significance attributed to magnesium supplementation is actually the effect of the interaction between magnesium and fluoride, since magnesium alone resulted in a very slight increase in growth whereas supplemental magnesium in the presence of fluoride caused a marked reduction in growth. Thus, it would appear that fluoride alone reduced growth, that magnesium alone did not affect growth, and that the combination of these two elements depressed growth to a greater extent than fluoride alone.

Approximately 6 days after the chicks had been placed on treatment, it was observed that many of the chicks in lot 4 (plus Mg and F) were reluctant to stand or walk and exhibited a characteristic leg weakness. When disturbed, the chicks walked on their hocks. This syndrome appeared to improve with time although no change was made in the diet.

TABLE 1

Interrelationships between magnesium and fluoride

Lot number	1	2	3	4
Supplemental fluoride, %	—	0.08	—	0.08
Supplemental magnesium, %	—	—	0.25	0.25
Av. 4-week weights, gm[1]	524	434	531	359
Mortality, %	0.0	0.0	0.0	13.3
Plasma inorganic phosphorus[2]	6.10	5.94	6.62	6.52
Plasma magnesium[2]	2.31	2.45	3.12	3.48

[1] Average of three replicates of 15 birds each.
[2] Average of 10 individual determinations.

TABLE 2

Interrelationships between magnesium, fluoride and phosphorus

Lot number	5	6	7	8	9	10	11	12
Phosphorus, %	0.70	0.70	0.70	0.70	1.0	1.0	1.0	1.0
Supplemental fluoride, %	—	0.08	—	0.08	—	0.08	—	0.08
Supplemental magnesium, %	—	—	0.25	0.25	—	—	0.25	0.25
Av. 4-week weights, gm[1]	571	514	565	457	590	509	578	475
Mortality, %	0.0	0.0	0.0	3.3	3.3	6.7	0.0	16.7
Plasma inorganic phosphorus[2]	6.56	7.18	6.40	6.66	6.08	6.93	6.94	7.44
Plasma magnesium[2]	2.87	2.06	2.91	3.19	2.26	2.58	3.77	3.38
Bone ash, % dry, fat-free bone[2]	41.74	44.01	41.96	35.49	42.37	42.09	41.44	38.18
Bone Ca, % dry, fat-free bone[2]	15.16	14.91	14.52	12.40	14.92	14.51	14.65	13.28
Bone Ca, % bone ash[2]	36.29	34.32	33.72	34.95	35.21	34.54	35.52	33.25
Bone P, % dry, fat-free bone[2]	7.47	7.85	7.55	6.19	7.45	7.30	7.36	6.75
Bone P, % bone ash[2]	18.23	18.02	17.61	17.44	17.61	17.33	17.88	17.77

[1] Average of three replicates of 10 birds each.
[2] Average of 5 individual determinations.

Supplemental dietary magnesium raised the plasma magnesium level significantly ($P < 0.01$), but fluoride did not appear to affect plasma magnesium. The differences in plasma inorganic phosphorus levels between the various groups were not statistically significant.

Experiment 2. In view of the retarded growth and leg weakness observed in the preceding experiment resulting from the addition of magnesium and fluoride to the diet, this experiment was designed with the following three main objectives: (1) to verify the earlier findings; (2) to study the possible relationship between the leg weakness syndrome and an alteration in calcification of the bone; and (3) to observe the effect of increasing the phosphorus content of the diet on the magnesium-fluoride interrelationship. It was believed that the leg weakness might have been associated with a reduction in bone calcification resulting from a deficiency of available phosphorus brought about by the formation of magnesium fluorophosphate, even though inorganic phosphorus content of the plasma was not affected by treatment in experiment 1. The results are outlined in table 2.

The growth data agree favorably with that in experiment 1. Growth was depressed significantly as a result of supplemental magnesium and fluoride ($P < 0.01$), and the magnesium $\times$ fluoride interaction was significant ($P < 0.05$). As in the previous study, fluoride alone reduced growth; magnesium alone did not appear to affect growth appreciably, but a combination of both elements reduced growth to a greater extent than fluoride alone. These observations were noted on both levels of phosphorus, and phosphorus content of the diet did not significantly affect growth, nor were there any significant interactions between phosphorus and the other two elements.

The leg weakness which was noted in experiment 1 was also observed in this experiment. It was first observed after the birds had been on treatment for 5 days and most of the birds receiving the supplemental fluoride and magnesium were affected regardless of the phosphorus level of the ration (lots 8 and 12). The leg weakness gradually improved until at 4

weeks the majority of the birds did not show the syndrome.

Bone ash was reduced significantly by fluoride ($P < 0.05$) and by magnesium ($P < 0.01$) and the magnesium × fluoride interaction was significant ($P < 0.01$). A comparison of treatment means, however, would suggest that the significance due to fluoride and magnesium shown by analysis of variance is not the result of the addition of magnesium or fluoride per se, but is actually a result of the interaction between these two elements, which is similar to the significant effect on growth attributed to supplemental magnesium. Thus, neither fluoride nor magnesium alone seemed to affect bone ash, but when both fluoride and magnesium were included in the diet simultaneously, a marked reduction in bone ash occurred. Phosphorus had no effect on bone ash, but a significant phosphorus × magnesium × fluoride interaction ($P < 0.05$) was noted, which apparently resulted from the somewhat higher bone ash found with the 1.0% of phosphorus with supplemental fluoride and magnesium (lot 12) as compared with the 0.7% level (lot 8). The calcium and phosphorus content of the bone, calculated as a percentage of the dry, fat-free bone, paralleled the bone ash data for the most part. The calcium and phosphorus content of the bone ash differed very little between treatments.

A significant increase ($P < 0.01$) in plasma magnesium level was obtained by the addition of 0.25% of magnesium in the diet. Supplemental fluoride did not significantly influence the level of magnesium in the plasma. These results confirm the observations made in the preceding experiment on plasma magnesium. The phosphorus level of the diet failed to significantly influence the plasma magnesium level; however, a significant ($P < 0.05$) phosphorus × magnesium interaction and a highly significant ($P < 0.01$) phosphorus × magnesium × fluoride interaction was observed. No explanation is offered for these interactions.

The inorganic phosphorus content of the plasma was not significantly affected by any of the nutritional treatments.

The results obtained in the two experiments are quite consistent and demonstrate a rather dramatic interrelationship between magnesium and fluoride. The retardation in growth observed by the addition of 0.08% of fluoride agrees with the findings of Gardiner et al. ('59a, b) with this level of fluoride in the form of sodium fluoride. Although supplemental magnesium alone (0.25%) did not appear to be detrimental, the inclusion of both 0.08% of fluoride and 0.25% of magnesium in the diet reduced growth to a greater extent than fluoride alone, and resulted in leg weakness, reduced bone ash, reduced bone calcium and reduced bone phosphorus. None of these effects on bone development could be demonstrated with either supplemental fluoride or magnesium alone. This interrelationship between magnesium and fluoride on calcification *in vivo* agrees with the work of Goldenberg and Sobel ('51) in which inhibition of calcification by fluoride *in vitro* required the presence of magnesium. The addition of 0.25% of magnesium to the diet increased plasma magnesium, and therefore, presumably increased the inhibition by fluoride. It is possible that higher levels of fluoride might accomplish the same effect on bone formation, although the leg weakness syndrome has not been described on fluoride levels in excess of 0.08%. It cannot be stated that the effect of fluoride and magnesium on growth is mediated entirely by interfering with calcification, but it could be a result of inefficient utilization of carbohydrate by inhibiting enolase; in this case the results would agree with Warburg and Christian ('42) who reported that increasing the magnesium content of the media decreased the quantity of fluoride needed to inhibit enolase. In any event, it has been demonstrated quite clearly that an interrelationship exists between magnesium and fluoride. These results with chicks are not in accord with the work of Schuck ('38) and Dibak and Ginter ('59) with rats, and the reason for this discrepancy is not apparent, but might be the result of species differences or differences in quantities of fluoride and magnesium used.

SUMMARY

Two factorial experiments were conducted with chicks to investigate a possible interrelationship between high levels

of dietary fluoride and magnesium. The first experiment consisted of two levels of supplemental fluoride (none and 0.08%) and two levels of supplemental magnesium (none and 0.25%). The second experiment included the same levels of fluoride and magnesium duplicated on two levels of dietary phosphorus (0.7 and 1.0%). The following observations were made:

1. A level of 0.08% of supplemental fluoride from sodium fluoride reduced growth rate, but did not appear to affect bone ash, bone calcium, bone phosphorus, plasma magnesium or plasma inorganic phosphorus.

2. A level of 0.25% of supplemental magnesium from magnesium carbonate increased plasma magnesium, but did not influence any of the other variables measured.

3. The inclusion of 0.08% of fluoride and 0.25% of magnesium in the same diet caused a greater depression in growth than fluoride alone. Furthermore, the addition of both fluoride and magnesium to the diet resulted in a characteristic leg weakness, reduced bone ash, and a reduction in both the calcium and phosphorus content of the bone. The leg weakness and bone changes were not observed when the fluoride or magnesium was added singly to the diet.

4. Increasing the phosphorus content of the diet from 0.7 to 1.0% was without effect in altering the toxicity of fluoride or the magnesium × fluoride interrelationship.

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Sulfur-Containing Amino Acids in the Nutrition of the Saw-Toothed Grain Beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera:Silvanidae)[1]

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Information regarding sulfur-containing amino acids with reference to insects has been published recently (Gilmour, '61). Previous work (Davis, '56) suggested that L-cystine was required in the diet of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.), for optimal growth rate. This is a report on the role of cysteine, methionine, and related amino acids in survival and development of this insect.

EXPERIMENTAL

The composition of the diet used in these investigations is given in table 1. This diet differs from previous diets used in this work in that L-cystine was replaced by L-cysteine at twice the concentration to maintain sulfur and amino nitrogen levels, because cystine consists essentially of two cysteine molecules. The effects of 5 levels of L-cysteine and of 8 levels of L-methionine were investigated by the method previously described (Davis, '59). As the cysteine or the methionine was reduced, however, it was replaced by additional tryptophan, rather than by glycine, to maintain the nitrogen level of the diet. Glycine was not used as a replacement for withdrawn amino acids because it is convertible to serine, which in turn enters into the intermediary metabolism of methionine and cysteine (Meister, '57). Because glycine and serine are implicated in the metabolism of the sulfur-containing amino acids (Umbreit, '54; Meister, '57), investigations were also carried out to determine whether these amino acids were of importance in cysteine and methionine metabolism.

RESULTS

The effects of a progressive withdrawal of L-cysteine from the diet of *O. surinamensis* are recorded in table 2. The number of larvae pupating and the number of adults emerging were not affected by a reduction in the cysteine content of the diet. The development time to the pupal stage, however, was shortest with 0.12% of L-cysteine ($P < 0.01$), significantly longer with 0.01% to 0.06% of L-cysteine ($P < 0.05$), and longest without cysteine ($P < 0.05$). A similar relationship held with respect to total development time (table 2).

The results of reducing the L-methionine content of the diet were much different (table 3). Both the number of larvae pupating and the number of adults emerging were affected by a reduction in the methionine content of the diet. Adult emergence, however, was less affected than was pupation. Significantly more larvae pupated when the diet contained 0.56% of L-methionine ($P < 0.05$) than in any other case. With this concentration of methionine, the development time to the pupal stage was also significantly shorter ($P < 0.01$) than with any other concentration. At a level of 0.70% of L-methionine, growth and survival were no better than that obtained with concentrations of 0.03 to 0.14%.

This suppression of survival to the pupal stage with large quantities of methionine was also noted when L-cysteine or L-cysteine and glycine were replaced by an equivalent amount of L-methionine in addition to the optimal concentration of 0.56% (table 4). Glycine or L-serine, or both, sustained limited survival and development in the absence of cysteine and me-

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TABLE 1

Composition of chemically defined diet for Oryzaephilus surinamensis (L.)

Amino acids[1]	%	Other components[1]	%
L-Alanine	0.07	Bacteriological dextrin, cp	77.15
L-Arginine·HCl	0.67	Cholesterol,USP	0.81
L-Aspartic acid	1.14	Yeast nucleic acid	0.48
L-Cysteine (free base)	0.12	Salt mixture[2]	1.62
L-Glutamic acid	3.74	Folic acid	0.02
Glycine (free of ammonia)	2.88	Choline chloride	0.05
L-Histidine (free base)	0.52	I-Inositol, NF	0.05
L-Isoleucine	1.21	*p*-Aminobenzoic acid, NF	0.005
L-Leucine (methionine-free)	1.62	D-Ca. pantothenate, USP	0.005
L-Lysine monohydrochloride	1.37	Niacin, USP	0.005
L-Methionine	0.56	Pyridoxine·HCl, USP	0.005
L-Phenylalanine	1.02	Riboflavin, USP	0.005
L-Proline	0.07	Thiamine·HCl, USP	0.005
L-Serine	1.10	Biotin	1×10^{-5}
L-Threonine	0.73	Vitamin B_{12}	12×10^{-5}
L-Tryptophan	0.21	Linoleic acid	0.54
L-Tyrosine	1.04	DL-α-tocopherol	0.054
L-Valine	1.25		

[1] Mann Research Laboratories, Inc., New York, except where otherwise noted.
[2] Salt Mix M-D no. 185 (McCollum-Davis), Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Pupation, emergence, and development period of 100 individuals of Oryzaephilus surinamensis (L.) reared with a chemically defined diet in which the concentration of L-cysteine was progressively reduced and replaced by a corresponding amount of L-tryptophan

Percentage of L-cysteine in diet	No. pupating	Av. time	No. emerging	Av. time
		hours		*hours*
0.12	80	560 ± 7[1]	70	700 ± 6[1]
0.06	70	583 ± 11	61	728 ± 10
0.03	70	597 ± 12	60	732 ± 11
0.01	73	617 ± 17	65	742 ± 11
0.00	89	626 ± 10	71	757 ± 10

[1] Standard error.

TABLE 3

Pupation, emergence, and development period of 100 individuals of Oryzaephilus surinamensis (L.) reared with a chemically defined diet in which the concentration of L-methionine was progressively reduced and replaced by a corresponding amount of L-tryptophan

Percentage of L-methionine in diet	No. pupating	Av. time	No. emerging	Av. time
		hours		*hours*
0.70	67	683 ± 13[1]	45	840 ± 17[1]
0.56	86	516 ± 6	78	634 ± 7
0.28	74	576 ± 10	61	697 ± 10
0.14	66	574 ± 10	54	705 ± 12
0.07	65	645 ± 14	44	785 ± 16
0.03	67	771 ± 17	52	900 ± 19
0.01	46	1179 ± 30	34	1310 ± 41
0.00	8	1308 ± 107	6	1504 ± 129

[1] Standard error.

TABLE 4

Pupation, emergence, and development time of 100 individuals of Oryzaephilus surinamensis (L.) reared on a chemically defined diet in which sulfur-containing and related amino acids were replaced by equivalent amounts of amino acids operative in the metabolism of sulfur-containing amino acids

Amino acid(s) replaced	Replaced by	No. pupating	Av. time	No. emerging	Av. time
			hours		*hours*
L-Cysteine	L-tryptophan	89	626 ± 10[1]	71	757 ± 10[1]
L-Cysteine	glycine	86	606 ± 8	69	740 ± 9
L-Cysteine	L-methionine	62	666 ± 8	52	780 ± 12
L-Methionine	L-tryptophan	8	1308 ± 107	6	1504 ± 129
L-Methionine	glycine	4	1476 ± 136	1	1332
L-Methionine	L-cysteine	6	1512 ± 127	3	1660 ± 225
L-Cysteine and glycine	L-methionine	5	1015 ± 97	5	1150 ± 117
L-Methionine and glycine	L-cysteine	0	—	0	—
L-Cysteine and L-methionine	glycine	36	837 ± 21	33	968 ± 20
L-Methionine and L-serine	L-cysteine	1	1380	1	1524
L-Cysteine, L-methionine and glycine	L-serine	20	1112 ± 35	12	1264 ± 48
L-Cysteine, L-methionine and L-serine	glycine	26	987 ± 32	18	1107 ± 40
L-Methionine, glycine and L-serine	L-cysteine	0	—	0	—

[1] Standard error.

thionine (table 4). When L-cysteine and L-methionine were replaced by an equivalent amount of glycine in the presence of L-serine, survival and development were better than when these amino acids were replaced by equivalent amounts of L-serine or glycine in the absence of the other.

DISCUSSION

Cystine is usually considered a dispensable amino acid (West and Todd, '55; Meister, '57). However, it accelerates growth in mammals (Rose, '38; Lafon, '39) and in the saw-toothed grain beetle (Davis, '56). It is required for pupation (Singh and Brown, '57), adult emergence (Golberg and De Meillon, '48), and optimal egg production (Dimond et al., '56) of the yellow-fever mosquito, *Aedes aegypti* (L.). The results of the present investigation show that L-cysteine can replace L-cystine in the diet of the saw-toothed grain beetle. They also indicate that L-cysteine may be withdrawn completely from the diet without adverse effects other than a lengthening of the development period.

Most animals require a dietary source of methionine (West and Todd, '55), but the German cockroach, *Blattella germanica* (L.), can dispense with it (House, '49). The present studies indicate that methionine is required in the diet of *O. surinamensis* and that the rate of growth and the survival are proportional to the concentration of methionine up to a level of 0.56%. Above this level, both survival and growth rate were adversely affected. Retardation of the growth rate by excess methionine has been noted previously in the white rat (Russell et al., '52) and in the dog (Gessert and Phillips, '56).

It is generally stated that methionine can replace cystine in the diet. The saw-toothed grain beetle can dispense with cysteine with only a resultant slowing of the growth rate. But if equivalent amounts of methionine, in addition to the optimal concentration, are used to replace cysteine in the diet, the growth rate is slowed even further and the number surviving to the pupal stage is reduced considerably (table 4).

Because glycine cannot replace methionine in the presence of L-cysteine, but can replace it partially in the absence of L-cysteine (table 4), the results indicate that L-cysteine is toxic to larvae of the saw-toothed grain beetle and that this toxicity is overcome by the inclusion of L-methionine in the diet. L-Cysteine is also toxic

to rats reared with chemically defined diets when it is present in certain concentrations (Birnbaum et al., '57).

Although L-serine or glycine were able to replace L-methionine in the diet to only a limited extent in the absence of L-cysteine, this may indicate that insufficient quantities of these amino acids were present to fulfill the methionine requirements. These larvae with intracellular symbionts (Koch, '31) are probably capable of synthesizing sulfur-containing amino acids from the inorganic sulfur in McCollum-Davis's salt mixture as do the nymphs of cockroaches and the adults of the Japanese beetle (Henry and Block, '60; Haines et al., '60).

SUMMARY

The effects of the sulfur-containing and related amino acids on the survival and growth rate of saw-toothed grain beetles were investigated with a chemically defined diet. L-Cysteine can replace cystine in the diet of this organism. It is toxic to the saw-toothed grain beetle, but this toxicity can be reversed by including L-methionine in the diet. High concentrations of L-methionine in the diet are also deleterious to the organism. The optimal concentration in the diet is in the neighborhood of 0.56% of L-methionine. L-Methionine can be replaced, however, at least partially, by either L-serine or glycine in the absence of L-cysteine.

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Nutritional Studies with the Guinea Pig

VII. NIACIN

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Dietary studies have been conducted in this laboratory to determine the requirement of the guinea pig for various members of the vitamin B complex. The present report is concerned with niacin.

Earlier studies by other investigators (Harris, '39) on the niacin requirement of the guinea pig were inconclusive because the diets used were deficient in vitamins other than niacin. More recent work conducted in this laboratory shows that the growth of very young animals consuming a diet deficient in niacin was retarded and symptoms of deficiency developed although they were not the same as those observed in other animal species (Reid, '54, '57). Fabianek ('54) fed guinea pigs a purified diet deficient in niacin. Five of the 7 experimental animals developed deficiency symptoms and two of them died. Since none were fed the diet plus added niacin it is difficult to determine whether these animals had an uncomplicated niacin deficiency. In another study, however, with older animals in which the niacin was not withheld until the animals had been fed the diet for from 45 to 126 days, no sign of deficiency developed and no change was seen at autopsy. Sullivan and Strong ('58) produced an apparently acute type of niacin deficiency in guinea pigs by the daily subcutaneous injection of 1 to 2 mg of 6-amino-nicotinamide, a known niacin antagonist. This treatment resulted in loss of weight on the second day with diarrhea followed by death on the 5th to the 7th day. When 5 mg of nicotinamide were given simultaneously the animals appeared healthy but showed a loss of righting ability when placed on their backs.

The present study concerns (1) the niacin requirement of the guinea pig when fed different types and levels of protein and (2) the relation of tryptophan to the niacin requirement.

METHODS

Procedures described previously (Reid and Briggs, '53) were followed as to type of guinea pig,[1] age, sex, initial weight, and care of the animals. The amount and type of protein and level of niacin in the semisynthetic diet (no. 13, Reid and Briggs, '53) was modified as indicated in the tables. Starch was substituted for protein when the latter was lowered. Studies were also made of the ability of tryptophan to replace niacin in the above diets.[2] The tryptophan and niacin content of the casein and purified soybean proteins was determined microbiologically.[3]

RESULTS

Tryptophan values of 1.30 and 1.08% were obtained for the casein and soybean proteins, respectively. The niacin values determined were 0.587 and 2.55 μg per gm by acid hydrolysis and 0.60 and 2.65 μg per gm by alkaline hydrolysis in the two proteins, respectively.

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[1] Male guinea pigs, Hartley strain, three to 4 days old with a weight range of 95 to 115 gm.

[2] The requirement of the guinea pig for tryptophan with diets containing ample niacin is reported in another paper (Reid and Von Sallmann, '60). (No. VI of this series.)

[3] For the tryptophan determinations 80 cm^3 of 5 N KOH was added to 5 gm of protein, the mixture autoclaved at 120°F for 5 hours at pH 4.5, after which the volume was adjusted to 200 cm^3 and the pH to 6.8 (Krehl et al., '46a). Correction was made for the racemization of the tryptophan. The free niacin was determined after acid hydrolysis (1 N H_2SO_4, pH 4.5), autoclaving for 30 minutes at 120°, then filtering and adjusting pH to 6.8. Total niacin (free plus bound) was determined after alkaline hydrolysis (1 N KOH, pH 4.5), filtration and adjustment of pH to 6.8.

TABLE 1

Effect of niacin supplements on growth and survival of guinea pigs fed purified diets containing 30 or 20% of protein

Niacin	No. of experiments	No. of animals	No. of survivors	Average weights[1] Weeks fed diet		
				2	4	6
mg/100 gm				*gm*	*gm*	*gm*
			30% Casein			
None	3	17	8	145 ± 14[2]	172 ± 39	235 ± 56
0.5	2	14	11	148 ± 23	197 ± 39	260 ± 71
1.0	1	7	7	145 ± 5	231 ± 12	328 ± 13
2.0	3	20	20	152 ± 20	244 ± 27	334 ± 30
			30% Purified soybean protein[3]			
None	2	15	15	148 ± 15	251 ± 21	351 ± 22
0.5	1	5	5	149 ± 11	253 ± 13	372 ± 22
1.0	1	7	7	152 ± 15	236 ± 35	366 ± 32
			20% Casein + 1% L-arginine + 0.25% DL-methionine[4]			
None	2	8	0	143 ± 4	150	—
0.5	2	16	5	144 ± 12	231 ± 37	240 ± 34
1.0	2	8	7	165 ± 19	262 ± 31	342 ± 31
2.0	2	8	7	166 ± 16	256 ± 29	345 ± 39
5.0	2	6	6	175 ± 19	274 ± 20	361 ± 25
			20% Purified soybean protein + 0.5% DL-methionine[4]			
None	3	14	0	137 ± 7	—	—
1.0	2	12	11	152 ± 14	233 ± 30	307 ± 40
2.0	2	12	12	160 ± 14	262 ± 21	353 ± 23
5.0	2	12	12	165 ± 19	273 ± 31	362 ± 29
10.0	3	16	16	150 ± 13	254 ± 18	352 ± 36

[1] The weights at any given time in this and the other tables represent the weights of all animals surviving at that time.
[2] Standard deviation.
[3] ADM Assay Protein C-1, Archer-Daniel-Midland Company, Cincinnati.
[4] These amino acid supplements are necessary to insure good growth.

With no added niacin, 8 of the 17 animals in three experiments with a diet containing 30% of casein survived the 6-week experimental period (table 1). There was marked variability in the condition of the survivors. Some of them gained weight and it appeared that they might survive for an indefinite period, whereas others were definitely on the decline. As compared to the control animals (2 mg of niacin per 100 gm of diet) and in agreement with results previously reported (Reid, '54), all showed retardation of growth, paleness of feet, nose and ears, drooling, soiled coats, decreased appetites, and a tendency to diarrhea. Those most severely affected lost weight and showed a slightly increased tendency to prolapse of the rectum.[4] Neither dermatitis nor oral lesions were observed. The animals surviving for 6 weeks had an average weight of 235 ± 56 (SD) gm as compared with 334 ± 30 gm for the controls. When a supplement of 1% of L-arginine hydrochloride or of 0.5% of DL-methionine was added to the diet lacking niacin, growth was not improved and survival rate was decreased.

Blood studies in two of the three experiments with no added dietary niacin showed the following average values and standard deviation in deficient and control animals, respectively: hematocrit (%), 36.6 ± 2.2 and 42.7 ± 0.37; hemoglobin (gm per 100 ml), 12.1 ± 0.8 and 14.5 ± 0.28; erythrocytes (cells × 10^6 per mm^3), 5.23 ± 0.46 and 6.04 ± 0.38; leucocytes (cells per mm^3), 3150 ± 570 and 4570 ± 500. These differences are significant particularly with respect to hemoglobin and hematocrit val-

[4] This condition is occasionally observed in guinea pigs reared with this diet (no. 13) even when it is complete in all known essentials.

ues since there was no overlapping of individual values in the two groups.

The addition of 0.5 mg of niacin to 100 gm of the 30% casein diet improved survival and in some animals increased the weight. Seven of the 11 survivors attained a weight after 6 weeks of 200 gm, two were over 300 gm and two were under 200 gm. One milligram of niacin added to 100 gm of diet produced 100% survival and increased weight markedly. None weighed less than 200 gm and all except two weighed over 300 gm. In fact, the average weight was essentially the same as that obtained with higher levels of niacin. There was no evidence of a toxic effect of niacin with levels as high as 50 mg per 100 gm of diet.

The ration containing 30% of purified soybean protein even in the absence of added niacin produced better growth than the optimal casein ration (table 1). Despite the good growth performance, some of the animals receiving no added niacin had soft and poorly formed feces and a matting of the fur around the anus. The addition of only 0.5 mg of niacin per 100 gm of diet tended to correct this condition. Results of additional experiments not shown in the table showed that no significant variations in weight occurred with added levels of niacin up to 2 mg per 100 gm.

Tests were also conducted with 20% levels of both casein and the soybean protein. Previous studies[5] showed that maximal growth could be obtained with this diet containing 20% of casein plus 1% of L-arginine hydrochloride. To prevent a possible methionine deficiency, 0.25% of DL-methionine was also added. When no niacin was added to the amino acid-supplemented casein diet only one of the 8 guinea pigs was alive after 4 weeks and it succumbed shortly thereafter (table 1). The addition of 0.5 mg of niacin per 100 gm of diet resulted in a definite improvement in survival and growth but the latter was not maximal. Increasing the niacin to one milligram resulted in maximal growth. After three weeks, there was one death, the cause of which was not determined. No further improvement in growth occurred with the niacin at a level of 2 mg per 100 gm. One death occurred in the 2-mg group as a result of prolapse of the rectum.

With a 20% level of purified soybean protein supplemented with 0.5% DL-methionine[6] and no added niacin there were no survivors at the end of 4 weeks (table 1). The addition of 1 mg of niacin per 100 gm of diet resulted in the survival of 11 of the 12 experimental animals after 6 weeks. Four of these weighed less than 300 gm. With 2 mg of niacin per 100 gm of diet there was 100% survival and growth was better than when supplying 1 mg. None of the survivors weighed less than 300 gm and 7 attained a weight greater than 350 gm. Further additions of niacin, up to 10 gm per 100 gm, resulted in no further increase in weight.

To study the effect of tryptophan on the niacin requirement, diets containing 20% of protein were used since they produced the most severe tryptophan deficiency. With no added tryptophan there was only one survivor with the casein diet and none with the soybean protein diet at 6 weeks (table 2). The addition of only 0.1% of DL-tryptophan[7] resulted in some improvement in survival in both groups. The addition of 0.25% of DL-tryptophan produced 100% survival and good growth in both the casein and soybean protein groups, but the rate was higher in the casein group (6.6 gm versus 5.4 gm per day). Increasing the tryptophan supplement to 0.4% resulted in no further gain with either group.

A possible explanation for the difference in growth and survival of the guinea pigs fed the casein and soybean protein diets with no added niacin might involve the differential availability of the tryptophan in the two proteins. As previously stated, the casein contained 1.30% of tryptophan and the soybean protein, 1.08%. To test the comparative availability of the tryptophan in the two proteins, diets were prepared in which 20% of the protein was

[5] Reid, M. E. 1956 Protein and amino acid studies with the guinea pig. Federation Proc., 15: 570 (abstract).

[6] See footnote 5.

[7] D-Tryptophan has been shown (Reid and Von Sallmann, '60) to have not more than from one-fourth to one-third the growth-promoting activity of the L-form in the guinea pig. It is doubtful that it has any value in protecting the eyes against cataracts.

TABLE 2

Effect of tryptophan added to diets containing 20% of protein and no supplemental niacin on growth and survival of guinea pigs

Added DL-tryptophan	No. of experiments	No. of animals	No. of survivors	Average weights, Weeks fed diet: 2	4	6
%				*gm*	*gm*	*gm*
	20% Casein + 1.0% L-arginine[1] HCl + 0.25% DL-methionine[1]					
None	3	14	1	137±11	144±45	262
0.1	1	5	2	146±10	186±17	273
0.25	1	5	5	151±16	264±27	377±45
0.4	1	5	5	152±18	258±30	372±42
	20% Purified soybean protein + 0.5% DL-methionine[1]					
None	3	14	0	137±7	—	—
0.1	3	17	10	142±13	187±40	238±56
0.25	4	20	20	152±11	251±23	328±28
0.4	4	20	20	155±10	252±19	329±18

[1] Supplements necessary with this level of this protein to insure a good rate of growth.

TABLE 3

Effect on growth and survival of diets having no added niacin and with amino acids partially replacing the protein

Diet with no added niacin	No. of animals	No. of survivors	Average weights, Weeks fed diet: 2	4
			gm	*gm*
30% Casein	28[1]	17(3)[2]	142±16	193±29
10% Casein + 20% amino acids	27[1]	24(6)[2]	150±17	212±25
30% Soybean protein	20	20	144±10	237±23
10% Soybean protein + 20% amino acids	20	18(1)[2]	144±13	203±27

[1] Thirty animals at start of experiment; a few failed to eat the diet well and died early without showing definite evidence of a dietary response.

[2] Number of animals which had prolapse of the rectum indicated in parentheses.

replaced with a mixture of amino acids in the proportions in which they occur in the protein. Ten per cent of casein or soy protein was added to make a total content of protein and amino acids in each of the two diets equal to that in 30% of the protein. One per cent of calcium carbonate was added to the diets containing amino acids but even with this addition the diets had a pH of 5.7[8] and were not as palatable as the diets containing 30% of protein (pH 7.9). The animals were maintained with these diets for 4 weeks. Results of the test are shown in table 3. Eight of the 28 animals fed the 30% casein diet died of niacin deficiency whereas only three of 27 animals receiving the amino acid diet died and these had prolapse of the rectum. The differences in growth of the two groups were small, although only one-third of the animals fed the 30% casein diet attained a weight of 200 gm or over as compared with two-thirds receiving the amino acid diet. The ears and toes of the animals fed the 30% casein diet tended to be pale as compared with those supplied with the diet containing amino acids. In the latter the ready availability of tryptophan apparently resulted in an increased production of niacin. In agreement with

[8] Arginine, histidine, and lysine were added as hydrochlorides in the L-form and methionine, phenylalanine, threonine, valine, and isoleucine were added in the DL-form. In one test in which the diets were prepared with all of the added amino acids in the L-form, neither the palatability nor the growth appeared to have been improved. Judging by the results obtained in another study with an amino acid mixture replacing dietary protein, it is considered likely that better results might have been obtained in the present studies if most of the nonessential amino acids had been omitted. Such a diet would undoubtedly have been more palatable.

TABLE 4

Effect of niacin supplements on growth and survival of guinea pigs fed a diet (GP 21) the protein of which was composed of 10% soybean protein, 10% gelatin and a supplement of all of the essential amino acids except tryptophan and arginine

Niacin	No. of experiments	No. of animals	No. of survivors	Average weights / Weeks fed diet		
				2	4	6
mg/100 gm				*gm*	*gm*	*gm*
None	3	15	0	123±10	190±21	—
2.0	2	9	7	124±10	204±35	218±47
2.5	2	10	10	146±16	218±23	292±32
10.0	3	13	11	140±18	222±38	305±39
20.0	8	40	36	148±13	228±27	301±36
20.0 + 0.1% L-tryptophan	2	10	10	160±26	260±20	339±21
50.0	4	20	15	138±12	187±26	238±41
50.0 + 0.15% DL-tryptophan	2	10	10	170±17	250±36	320±34

the results in the previous experiments, the animals fed the 30% soybean protein diet grew well without added niacin, and no deaths occurred. Substitution of 20% of the soybean protein with amino acids did not improve growth and two deaths occurred in a total of 20 animals. As with the casein diet, the tendency to prolapse of the rectum was increased somewhat by the substitution of amino acids for two-thirds of the protein.

To obtain further information on the tryptophan and niacin requirements of the guinea pig, experiments were conducted with a diet (GP 21)[9] containing 10% of the purified soybean protein and 10% of gelatin plus a mixture of all the essential amino acids except tryptophan and arginine. The diet contained 0.108% of L-tryptophan. The results obtained with this diet are shown in table 4. The addition of niacin had a beneficial effect on both growth and survival but maximal growth was not obtained with the addition of niacin only. With the addition of tryptophan, however, maximal growth was achieved. Varying degrees of cataractous change were observed in the eyes of the animals receiving no added tryptophan. Previous studies showed that this diet supplemented with adequate niacin does not meet the guinea pig's requirement for tryptophan (Reid and Von Sallmann, '60). The minimal requirement for niacin with this diet was found to be 2.5 mg per 100 gm. With the addition of a very high level of niacin (50 mg per 100 gm) poor growth and survival were obtained. Essentially the same results were noted in each of the 4 separate trials in this series. The depression in growth and survival observed with 50 mg of niacin per 100 gm of diet was corrected by the addition of 0.15% of DL-tryptophan.

DISCUSSION

The diet with 30% of soybean protein and no added niacin supported a good rate of growth and 100% survival but with casein as the protein at this level both growth and survival were poor. The growth produced with these diets should therefore be a measure of the rate of conversion of tryptophan to niacin. Since under these conditions almost maximal growth was obtained with the soybean

[9] The ration (GP 21) consisted of the following in percentage amounts: purified soybean protein (ADM C-1 Assay Protein, Archer-Daniel-Midland Company, Cincinnati), 10; gelatin, 10; corn oil, 7.3; sucrose, 10.2; Cellophane Spangles (Rayon Processing Company, Pawtucket, Rhode Island), 15; cornstarch, 20; glucose (Cerelose, Corn Products Company, New York), 13.9; potassium acetate, 2.5; magnesium oxide, 0.5; salts (Briggs et al., '52), 6; choline chloride, 0.2; ascorbic acid, 0.2; inositol, 0.2; liberal amounts of the known vitamins as in diet 13 (Reid and Briggs, '53); and an amino acid mixture, 4.0. The amino acid mixture contained the essential amino acids except tryptophan and arginine plus three of the nonessential amino acids. The formula for the mixture had the following composition (gm per 100 gm of diet): L-cystine, 0.04; L-glutamic acid, 0.43; L-histidine·HCl, 0.13; DL-isoleucine, 0.86; L-leucine, 0.34; L-lysine·HCl, 0.22; DL-methionine, 0.43; DL-phenylalanine, 0.26; DL-threonine, 0.43; L-tyrosine, 0.26; DL-valine, 0.60; total, 4.00.

protein but not with casein, the results suggest strongly that with the guinea pig a greater conversion of tryptophan to niacin occurs with the soybean protein even though the casein contains more tryptophan. With the 30% casein diet, 390 mg of tryptophan per 100 gm of diet are supplied and with the soybean protein, the tryptophan amounts to 324 mg per 100 gm. Previous studies (Reid and Von Sallmann, '60) have shown that the guinea pig requires approximately 200 mg of L-tryptophan per 100 gm of diet to satisfy its need for tryptophan as such (niacin supply adequate). As a consequence of this direct need of tryptophan for growth, at the 30% level of casein, 190 mg of L-tryptophan and with the soybean protein, 124 mg would become available for conversion to niacin. With 20% levels of protein in the diet the niacin requirement with casein as the protein is less because more tryptophan is available for conversion to niacin. The amounts of tryptophan available for conversion would be 60 mg per 100 gm of diet for casein and only 16 mg per 100 gm for the soybean protein. The difference between them of 44 mg represents the amount of tryptophan which is equivalent in value, according to the present data, to 1 mg of niacin. Since the casein supplied the tryptophan equivalent to 1 mg of niacin and 1 mg of added niacin was necessary to produce maximal growth, the total requirement of niacin amounted to 2 mg. With the soybean protein, 2 mg of added niacin was necessary because the amount of tryptophan available for conversion was too small to be of appreciable significance.

Information is available as to the niacin requirement with other types of dietary protein. An inhibitory effect on growth with gelatin in a niacin-deficient diet was observed in chicks by Briggs ('45) and in rats by Krehl et al. ('46b), Schweigert and Pearson ('48), and Salmon ('54). The inhibitory effect could be counteracted by supplementing the diet with either niacin or tryptophan. In the present studies, however, with the guinea pig, maximal growth could not be achieved with the soy protein-gelatin diet by the addition of niacin alone. Addition of tryptophan was also necessary. The low tryptophan level (0.108%) in this diet (GP 21) is believed to be responsible for the toxic effect of a very high level of added niacin (50 gm per 100 gm). The data in table 4 indicate that the toxicity can be counteracted by the addition of tryptophan to the diet. With the adequate level of dietary tryptophan, niacin at the level of 50 mg per 100 gm was not toxic with a diet containing 30% of casein (table 1). It was shown by Handler ('44) that as much as 1 or 2% of nicotinamide in the diet of the guinea pig did not depress growth.

The conversion of tryptophan to niacin has been studied by Henderson et al. ('49) who measured the efficiency of conversion of tryptophan to precursors of niacin. The percentage of the tryptophan which appeared as urinary quinolinic acid varied from approximately 0.2% for man and the guinea pig to 10 to 20% for the rat.

The differences herein obtained in the niacin requirement for growth of the guinea pig with different proteins at different levels of intake are in general agreement with results obtained with rats, cotton rats and mice by Schweigert and Pearson ('48). The guinea pig's requirement for niacin is low as compared with that of most other animals (Hundley, '54). It is much like that of the rat (Hundley, '54) and mouse (Schweigert and Pearson, '48) but much lower than that of the dog (Schaefer et al., '42), swine (Luecke et al., '47), and rabbit (Wooley, '47; Olcese et al., '49), and only slightly, if any, lower than that of the chicken (Briggs et al., '42, '46). It seems quite possible that in those animals with a requirement for niacin higher than that of the guinea pig, the requirement for tryptophan as such may also be higher.

The present results suggest the desirability of determining the growth requirement of an animal for tryptophan in the presence of ample niacin and of using the data derived therefrom in estimating the efficiency of conversion of tryptophan to niacin. Even with this procedure it will probably be difficult to obtain a high degree of accuracy because it is doubtful that niacin synthesis would be entirely suspended until all the requirements of tryptophan for growth were satisfied.

The results of the present experiments suggest that the type of dietary protein can exert a definite effect on the availability of tryptophan for conversion to niacin. The availability appears to be less with dietary casein than with the soybean protein. Gupta and Elvehjem ('57), in studies with the rat reported that the biological availability of tryptophan in purified soybean protein was slightly higher than in casein. Suggestive evidence is available from other work also that the conversion of the tryptophan in casein occurs relatively slowly. When tryptophan is supplied as the free amino acid, considerably greater synthesis of niacin occurs than if an equivalent amount of tryptophan is supplied as casein (Singal et al., '46; Bell et al., '48; Schweigert and Pearson, '48). In the present studies with diets having no added niacin, substitutition of two-thirds of the casein (30%) with a mixture of the amino acids found in casein gave better survival and less evidence of niacin deficiency than was noted with the 30% casein diet. The reason for this apparent difference in the ease of conversion of tryptophan to niacin in the two proteins is not clear. It is possible that a difference in protein structure may be involved whereby the tryptophan of the soybean protein is more readily accessible to enzyme action. Also, it is possible that the presence of somewhat greater trace amounts of niacin in the soybean protein may exert a slight influence.

SUMMARY

The young guinea pig requires a dietary source of niacin unless there is an ample supply of available tryptophan. Like other animals, it has the capacity to produce niacin from tryptophan.

The dietary requirement for niacin is affected by the amount and type of protein in the diet, particularly with respect to its tryptophan content. Maximal growth was obtained with a purified diet by the addition of 1 mg of niacin per 100 gm, either to a 30% casein diet or to one containing 20% of casein supplemented with 1% of L-arginine. With a diet containing 30% of purified soybean protein, maximal growth was obtained without addition of niacin, but with a 20% level of this protein supplemented with 0.5% of DL-methionine, more than 1 mg of niacin, possibly as much as 2 mg, was necessary. With a diet containing 10% of the soybean protein and 10% of gelatin, supplemented with the essential amino acids except tryptophan and arginine, the niacin requirement was found to be 2.5 mg per 100 gm. With this latter diet, maximal growth was not obtained without addition of tryptophan.

The conversion of tryptophan to niacin appears to occur more efficiently with the soybean protein than with casein.

Extremely high levels of niacin were somewhat toxic to the guinea pig when fed the gelatin-containing diet if the amount of available tryptophan was insufficient to supply the need for tryptophan as such.

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Influence of Graded Levels of Dietary Linoleic and Linolenic Acids on the Fatty Acid Composition of Hens' Eggs[1]

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Previous studies in this laboratory (Reiser, '50) indicated that there is no difference in the amount of moisture, total lipid, phospholipid or cholesterol in eggs from hens fed a low-fat or a 4% cottonseed oil diet. It is clear, however, that the fatty acid composition of the egg lipids depends upon the dietary fat (Cruikshank, '34). It has also been reported by Reiser ('51) that either a low-fat diet or one containing a saturated fat, such as bayberry tallow, results in the reduction of the dienoic acid content of egg yolk lipids. Other investigations (Cruikshank, '34; Reiser, '51; Feigenbaum and Fisher, '59) have shown that an increase in egg polyunsaturated fatty acids, upon their ingestion, takes place at the expense of oleic acid. It has also been reported (Feigenbaum and Fisher, '59) that when various oils are fed at the 10% level of the diet, the composition of the body fat is influenced only by the dietary polyunsaturated fatty acids, whereas the composition of the egg yolk fat is influenced by either saturated or unsaturated fatty acids in the diet. Choudhury and Reiser ('59) reported that the level of linoleic acid in the lipids of the egg is not a straight line function of the linoleic acid in the diet. Wheeler et al., ('59) showed that about 40% of linoleic acid and 14% of linolenic acid may be incorporated in the egg lipids when the hens are fed safflower and linseed oils, respectively.

The present investigation was designed to test the effect of graded levels of dietary linoleic and linolenic acids, fed alone and with tallow, on the levels of egg lipid fatty acids as determined by gas-liquid chromatography.

EXPERIMENTAL

Eight hens were fed a low-fat diet for 6 weeks. The composition of the diet is shown in table 1. After the hens consumed this diet for 6 weeks, the linoleic acid in the egg lipids reached a minimum level of about 3%, and the linolenic acid was reduced to trace quantities. At that time, relatively pure trilinolein[3] or trilinolenin[4] was fed with or without tallow, according to the schedules in tables 3, 4, 5, and 6. The trilinolein and trilinolenin were prepared from the free fatty acids by esterification with methanol and subsequent interesterification with stoichiometric quantities of triacetin in the presence of 0.5% sodium methoxide. Fatty acid composition of the dietary fats, as determined by gas-liquid chromatography, is presented in table 2. The hens were supplied with each dietary level until the egg fatty acid levels reached plateaus. This required approximately 15 days. At each level the 7th, 9th, 11th and 12th eggs were analyzed. The fatty acid composition of the 9th and 11th eggs was quite similar. The 12th was within 0.5% of the 11th. The next level was then added to the diet.

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[4] See footnote 3.

TABLE 1

Composition of basal low-fat diet

	gm/kg diet
Glucose monohydrate[1]	542.61
Purified soybean protein[2]	225.00
Woodpulp	30.00
Mineral mix[3]	35.08
Vitamin mix[4]	14.47
$CaHPO_4$	52.66
Choline chloride	2.00

[1] Cerelose, Corn Products Company, Dallas, Texas.

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

[3] Supplied the following per kilogram of diet: (in grams) oyster shell, 15.00; sodium chloride, 5.00; magnesium sulphate heptahydrate, 5.27; manganese sulphate heptahydrate, 0.15; ferrous sulphate heptahydrate, 0.011; cupric sulphate pentahydrate, 0.011; zinc chloride, 0.106; potassium iodate, 0.0129; cobalt chloride monohydrate, 0.016; potassium chloride, 7.63; and sodium molybdate monohydrate, 0.0076.

[4] Supplied the following per kilogram of diet: (in milligrams) thiamin·HCl, 10; D-calcium pantothenate, 20; pyridoxine, 6; *p*-aminobenzoic acid, 20; inositol, 500; biotin, 0.2; vitamin B_{12}, 20; vitamin D_3 concentrate (200,000 IU/gm), 10; niacin, 75; folic acid, 4; chlortetracycline hydrochloride, 10; butylated hydroxy toluene, 133; menadione, 5; riboflavin, 10; and (in grams) vitamin E, 167; DL-methionine, 7.50; glycine, 3.5; vitamin A concentrate (10,000 IU/gm), 1.0.

TABLE 2

Fatty acid composition of dietary fats[1]

Acids[2]	Trilinolein[3]	Trilinolenin[3]	Tallow[4]
	%	%	%
14:0	0.16	0.00	2.44
14:1	0.00	0.00	1.08
16:0	1.46	0.20	23.42
16:1	0.00	0.00	6.23
18:0	0.00	0.72	12.76
18:1	3.31	1.00	50.48
18:2	94.24	8.87	2.87
18:3	0.84	89.21	0.72

[1] Determined by gas chromatography on ethylene-glycol succinate polyester, 20%, on 60–80 mesh acid-washed celite. Column temperature 195°C, flow rate 60 ml of argon per minute ionization detector.

[2] Numbers before the colon represent the number of carbon atoms, and numbers after the colon represent the number of double bonds.

[3] Generously donated by Northern Utilization Research and Development Division, USDA, Peoria, Illinois.

[4] DAR-GLY, refined tallow obtained from Darling and Company, Chicago, Illinois.

Egg lipids were extracted from the yolk with chloroform in a homogenizer, and the triglycerides and phospholipids were separated by slurrying with silicic acid (Murty et al., '60). The fatty acid composition of the triglycerides and phospholipids fatty acids was obtained by gas-liquid chromatography of their methyl esters, which were prepared by saponification followed by acidification and esterification with diazomethane (De Boer, '54).

After being fed the unsaturated acids at a 5% level, each hen was injected with 0.2 mc of acetate-1-C^{14} intraperitoneally to determine the effect of the diets on the degree of incorporation of acetate into fatty acids and cholesterol. The radioactivity of the egg fatty acids and cholesterol from the most radioactive egg from each group was determined using a liquid scintillation spectrometer.[5] Cholesterol was isolated as the tomatinide (Kabara et al., '61) and aliquots were taken for assay by Zak's colorimetric method ('54) and for radioactivity.

RESULTS AND DISCUSSION

The fatty acid compositions of the egg triglycerides and phospholipids are presented in tables 3, 4, 5 and 6. In the absence of other dietary fat, the levels of linoleic and linolenic acids in the egg lipids were dependent upon the dietary levels up to about 5%. Above 5% in the diet there were no further increases in their levels of deposition in the egg. The addition of tallow to a total of 10% of dietary fat resulted in a reduction in the levels of linoleic or linolenic acids in the egg lipids at all levels in the diet. Thus, at 5% in the diet with 5% of tallow, the levels of linoleic acid in the egg triglycerides and phospholipids were only 11 and 12%, respectively, as compared with 24 and 18% when no tallow was included. Linolenic acid incorporation was depressed from maximal levels of 14% in triglycerides and 11% in phospholipids when fed at the 5% level without tallow, to 8 and 6%, respectively, with 5% of tallow. In a previous report from this laboratory (Choudhury and Reiser, '59), it was shown that 7.5% of linoleic acid is required to reach the maximal level of incorporation when fed as safflower oil. Therefore, a higher level of dietary linoleic and linolenic acids is re-

[5] Tricarb, Packard Instrument Company, La Grange, Illinois.

TABLE 3

Triglyceride[1] fatty acid composition of eggs of hens fed trilinolein diet with or without tallow[2,3,4]

Hen no.	Level of added fat in diet	14:0	14:1	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:2
		%	%	%	%	%	%	%	%	%	%
1	Low-fat	0.97	0.00	30.36	11.12	0.00	4.84	49.10	3.09	0.10	0.00
	Trilinolein, 0.25%	0.53	0.00	32.41	7.30	0.00	6.28	50.33	2.70	0.46	0.00
	Trilinolein, 0.50%	0.55	0.26	28.69	6.75	0.00	5.39	52.27	5.76	0.33	0.00
	Trilinolein, 0.75%	0.38	0.27	27.45	8.77	0.00	6.40	49.26	7.13	0.34	0.00
2	Low-fat	0.57	0.17	28.49	6.15	0.00	6.49	56.88	0.72	0.52	0.00
	Trilinolein, 1.50%	0.39	0.17	28.95	4.95	0.00	9.55	49.44	6.08	0.46	0.00
	Trilinolein, 3.00%	0.57	0.23	28.82	3.76	0.00	7.64	43.66	14.66	0.71	0.00
	Trilinolein, 5.00%	0.34	0.10	25.72	4.71	0.00	7.09	37.52	24.25	0.27	0.00
	Trilinolein, 7.50%	0.42	0.23	27.08	3.77	0.00	7.55	36.58	24.90	0.22	0.17
3	Tallow, 10.00%	0.51	0.31	24.97	4.09	0.30	6.30	61.86	0.92	0.68	0.00
	Tallow, 9.75% + trilinolein, 0.25%	0.49	0.31	26.65	3.91	0.00	4.68	61.12	1.86	0.92	0.00
	Tallow, 9.50% + trilinolein, 0.50%	0.55	0.43	25.70	2.31	0.00	5.16	61.83	3.23	0.71	0.00
	Tallow, 8.75% + trilinolein, 1.25%	0.44	0.25	25.12	4.74	0.00	4.72	58.20	5.45	0.78	0.00
4	Tallow, 10.00%	0.41	0.37	27.42	4.82	0.39	5.73	58.80	1.06	1.00	0.00
	Tallow, 7.20% + trilinolein, 2.80%	0.54	0.55	27.22	3.70	0.00	6.61	54.25	6.50	0.64	0.00
	Tallow, 5.15% + trilinolein, 4.85%	0.49	0.20	26.66	3.55	0.37	6.75	50.44	11.03	0.49	0.00

[1] Also includes cholesterol esters, free fatty acids and partial glycerides.

[2] These values are the plateau levels reached in the eggs after the changes in the diet. After reaching the plateau in the 11th or 12th egg, the diet was changed to the next level.

[3] Numbers before the colon represent the number of carbon atoms and the numbers after the colon represent the number of double bonds.

[4] 18:1 and 18:2 acids underwent the most significant changes.

TABLE 4

Triglyceride[1] fatty acid composition of eggs of hens fed trilinolenin diet with or without tallow[2,3,4]

Hen no.	Level of added fat in diet	14:0	14:1	16:0	16:1	16:2	18:0	18:1	18:2	18:3
		%	%	%	%	%	%	%	%	%
5	Low-fat	0.30	0.00	25.11	4.86	0.00	7.62	60.37	0.97	0.76
	Trilinolenin, 0.25%	0.33	0.00	24.10	4.82	0.00	6.42	64.24	0.84	0.94
	Trilinolenin, 0.50%	0.30	0.11	22.59	4.95	0.00	3.83	63.93	2.87	1.42
	Trilinolenin, 0.75%	0.43	0.37	22.97	7.43	0.00	5.79	56.37	3.90	2.74
6	Low-fat	0.45	0.19	26.79	5.12	0.00	6.43	59.42	0.96	0.64
	Trilinolenin, 1.50%	0.55	0.00	24.44	5.70	0.00	7.11	56.35	1.69	4.15
	Trilinolenin, 3.00%	0.28	0.12	24.43	6.03	0.00	6.76	51.74	2.84	7.80
	Trilinolenin, 5.00%	0.39	0.28	21.00	5.52	0.00	6.35	51.14	4.07	11.25
	Trilinolenin, 7.50%	0.00	0.00	22.49	7.00	0.39	6.59	46.75	4.62	12.06
7	Tallow, 1.0%	0.39	0.21	22.46	3.81	0.00	6.48	64.56	1.00	1.09
	Tallow, 9.50% + trilinolenin, 0.50%	0.33	0.31	20.13	3.65	0.83	5.24	64.24	2.94	1.92
	Tallow, 9.25% + trilinolenin, 0.75%	0.35	0.30	22.23	4.92	0.52	3.63	62.27	3.74	2.04
	Tallow, 8.50% + trilinolenin, 1.50%	0.52	0.27	22.36	5.94	0.00	8.88	52.66	2.53	2.84
8	Tallow, 10.0%	0.63	0.36	22.07	3.79	0.00	5.69	65.70	5.93	0.81
	Tallow, 7.00% + trilinolenin, 3.00%	0.34	0.19	25.33	3.69	0.22	6.51	56.85	3.01	3.86
	Tallow, 5.00% + trilinolenin, 5.00%	0.49	0.43	22.45	4.53	0.49	6.48	52.46	4.70	7.97

[1] Also contains cholesterol esters, free fatty acids and partial glycerides.

[2] These values are the plateau levels reached in the eggs after the changes in the diet. After reaching the plateau in the 11th or the 12th egg, the diet was changed to the next level.

[3] Numbers before the colon represent the number of carbon atoms and the numbers after the colon represent the number of double bonds.

[4] 18:1 and 18:3 acids underwent the most significant changes.

TABLE 5

Phospholipid fatty acid composition of eggs of hens fed trilinolein diet with or without tallow[1,2,3]

Hen no.	Level of added fat in diet	14:0	14:1	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:0	20:2	20:3	20:4	20:6	22:0	22:5
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	Low-fat	0.00	0.00	30.11	4.72	0.00	16.11	43.39	2.00	0.23	0.00	0.00	0.68	2.75	0.00	0.00	0.00
	Trilinolein, 0.25%	0.21	0.00	30.49	4.30	4.30	14.99	40.13	4.16	0.32	0.00	0.00	0.58	2.96	0.00	0.79	1.06
	Trilinolein, 0.50%	0.24	0.44	28.53	5.04	0.00	12.38	42.28	5.25	0.35	0.00	0.00	0.82	3.21	0.00	0.00	1.36
	Trilinolein, 0.75%	0.42	0.53	26.63	7.70	0.00	12.23	38.57	6.33	0.24	0.03	0.00	0.53	3.19	1.08	0.47	2.06
2	Low-fat	0.00	0.00	25.29	4.64	0.00	13.92	48.96	3.71	0.00	0.00	0.00	0.00	3.48	0.00	0.00	0.00
	Trilinolein, 1.50%	0.11	0.00	24.92	2.43	0.00	21.71	36.54	6.82	0.24	0.00	0.00	0.66	4.34	0.00	0.64	1.58
	Trilinolein, 3.00%	0.35	0.24	27.33	3.19	0.45	16.29	34.25	11.71	0.59	0.00	0.00	0.00	3.67	0.00	0.00	1.89
	Trilinolein, 5.00%	0.28	0.22	25.65	3.82	0.00	16.50	29.58	17.84	0.25	0.00	0.00	0.17	2.88	0.61	0.20	2.00
	Trilinolein, 7.50%	0.50	0.22	27.16	2.79	0.00	14.76	27.69	18.42	0.23	0.00	0.13	0.59	4.31	0.82	0.15	2.31
3	Tallow, 10.00%	0.54	0.22	25.67	8.06	0.14	8.83	52.92	1.25	0.60	0.00	0.80	0.45	1.24	0.00	0.00	0.00
	Tallow, 9.75% + trilinolein, 0.25%	0.32	0.35	24.80	3.38	0.00	11.19	53.64	2.88	0.70	0.00	0.00	0.15	1.77	0.00	0.54	0.00
	Tallow, 9.50% + trilinolein, 0.50%	0.33	0.19	23.40	4.27	0.00	10.09	53.17	4.36	0.73	0.00	0.00	0.36	1.76	0.28	0.38	0.61
	Tallow, 8.75% + trilinolein, 1.25%	0.51	0.38	24.80	3.97	0.00	9.18	51.04	6.13	0.70	0.00	0.00	0.00	2.27	0.00	0.00	1.02
4	Tallow, 10.00%	0.39	0.27	27.13	4.20	0.27	8.49	54.76	2.04	0.79	0.00	0.00	0.48	1.17	0.00	0.00	0.00
	Tallow, 7.20% + trilinolein, 2.80%	0.26	0.20	20.48	2.35	0.00	17.38	44.60	5.79	0.37	0.00	0.52	2.18	3.69	0.00	0.69	0.89
	Tallow, 5.15% + trilinolein, 4.85%	0.51	0.53	28.42	2.99	0.00	13.14	36.43	12.22	0.37	0.00	0.00	0.17	3.71	0.00	0.00	15.1

[1] These values are the plateau levels reached in the eggs after the changes in the diet. After reaching the plateau in the 11th or 12th egg, the diet was changed to the next level.

[2] Numbers before the colons represent the number of carbon atoms and numbers after the colon represent the number of double bonds.

[3] These acids underwent the most significant changes.

TABLE 6

Phospholipid fatty acid composition of eggs of hens fed trilinolenin diet with or without tallow[1,2,3,4]

Hen no.	Level of added fat in diet	14:0	14:1	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5	22:5	22:6
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
5	Low-fat	0.00	0.00	22.83	5.43	0.00	13.70	51.31	2.17	1.09	1.00	0.96	1.52	0.00	0.00	0.00
	Trilinolenin, 0.25%	0.16	0.00	23.65	3.81	0.00	15.34	49.55	2.57	0.68	0.00	0.28	1.78	0.00	0.00	1.10
	Trilinolenin, 0.50%	0.20	0.10	23.81	4.23	0.00	14.99	45.56	3.24	1.06	0.00	0.39	2.74	0.47	0.00	3.20
	Trilinolenin, 0.75%	0.31	0.31	23.71	6.31	0.00	10.99	47.73	3.96	1.86	0.00	0.26	1.35	0.59	0.00	2.38
6	Low-fat	0.39	0.16	22.54	2.80	0.00	15.23	53.04	1.50	0.67	0.00	0.23	1.94	0.00	0.00	0.00
	Trilinolenin, 1.50%	0.27	0.00	23.91	3.09	0.00	19.72	42.01	1.20	1.92	0.37	0.16	1.98	0.71	0.00	3.76
	Trilinolenin, 3.00%	0.25	0.25	24.77	3.58	0.00	14.64	43.93	2.56	4.40	0.00	0.15	1.05	1.19	0.00	3.23
	Trilinolenin, 5.00%	0.30	0.17	21.41	4.62	0.00	13.26	43.02	3.56	8.05	0.00	0.00	0.68	0.66	1.40	2.86
	Trilinolenin, 9.50%	0.38	0.30	21.95	4.84	0.00	14.60	39.71	4.20	8.72	0.09	0.00	0.89	1.38	0.51	2.44
7	Tallow, 10.00%	0.22	0.17	23.00	2.65	0.00	16.55	51.21	2.07	0.59	0.12	1.25	1.86	0.00	0.00	0.00
	Tallow, 9.50% + trilinolenin, 0.50%	0.17	0.16	22.05	2.93	0.12	10.39	58.99	1.44	0.90	0.11	0.37	1.12	0.00	0.00	1.24
	Tallow, 9.25% + trilinolenin, 0.75%	0.30	0.26	23.41	4.29	0.00	12.31	51.55	3.45	1.46	0.00	0.00	1.34	0.00	0.00	1.63
	Tallow, 8.50% + trilinolenin, 1.50%	0.34	0.35	27.07	4.21	0.00	10.71	48.37	2.79	2.20	0.00	0.32	0.87	0.69	0.00	1.65
8	Tallow, 10.00%	0.39	0.29	27.12	3.65	0.24	10.20	53.83	2.01	0.60	0.00	0.49	1.18	0.00	0.00	0.00
	Tallow, 7.00% + trilinolenin, 3.00%	0.29	0.28	23.22	3.26	0.54	11.36	48.59	3.46	3.90	0.00	0.00	1.17	0.52	0.88	2.53
	Tallow, 5.00% + trilinolenin, 5.00%	0.23	0.31	25.53	3.46	0.00	13.80	42.91	4.18	5.97	0.00	0.00	0.90	0.81	0.00	1.92

[1] These values are the plateau levels reached in the eggs after the changes in the diet. After reaching the plateau in the 11th or 12th egg, the diet was changed to the next level.

[2] Numbers before the colon represent the number of carbon atoms and the numbers after the colon represent the number of double bonds.

[3] Underscoring indicates the acids that underwent the most significant changes.

[4] Small and irregularly changing values in 20:6 and 22:0 are not included in the table.

quired in the diet to reach the maximal levels in the eggs when they are fed with another fat or as only part of a fat than when fed alone. In all probability this is due to dilution since some of each of the dietary fatty acids are incorporated into the egg. It had been anticipated that the ingestion of tallow would spare the utilization of the polyunsaturated acids and that more would be deposited. If such is the case, the dilution factor more than compensates for it because less polyunsaturated acids were deposited.

There is no obvious explanation for the much lower level of linolenic than linoleic acid in the egg lipids. As reviewed by Deuel ('54) it has long been observed that linolenic acid is deposited to a lesser degree than linoleic in animal fat. In part this could be due to desaturation or other interconversions as well as possible preferential utilization, but the reason is not known.

In all instances the deposition of linoleic and linolenic acids in the egg was mainly at the expense of oleic, both in triglycerides and phospholipids. Stearic acid, however, also is markedly decreased in phospholipids. The data indicate that when a fat containing stearic and oleic acids is added to a low-fat regimen, the stearic acid increases at first and then is decreased. It is a question whether this is due to a delay in the homeostatic mechanism or whether the sudden appearance of exogenous oleic acid inhibits the desaturation of stearic to oleic acid.

The phospholipid data show clearly the conversions of linoleic and linolenic acids to higher polyunsaturated acids. The graded increases in dietary linoleic acid were accompanied by increases in arachidonic and by the appearance and subsequent increases in the level of eicosahexaenoic and docosapentaenoic acids.

After linolenic acid ingestion, eicosapentaenoic and docosahexaenoic acids appeared. Docosahexaenoic acid increased in percentage quite rapidly and leveled off between 2 and 3%, whereas eicosapentaenoic acid was deposited at a lower level. Unpublished incomplete studies in this laboratory with marine fish also indicate that dietary linolenic acid is the origin of the large levels of docosahexaenoic acid present in those animals.

A significant change after the ingestion of linolenic acid is the decrease in the level of arachidonic acid. The explanation for this is not obvious. The appearance of docosapentaenoic acid is probably due to the presence of 9% of linoleic acid in the linolenic acid supplement.

These conversions are in accord with the principle of the elongation of unsaturated acids by alternative desaturation at the methylene-interrupted positions and by the addition of acetate (Mead and Howton, '58).

The mechanisms for handling the two important dietary polyunsaturated acids are very different, not only with reference to their degree of utilization and deposition, but also with respect to their influence on the synthesis, utilization and deposition of other acids, their different functions as "essential" fatty acids, and in their alleged roles in cholesterol metabolism.

The radioactivity of the egg fatty acids and cholesterol is presented in table 7. The fatty acid activity values are corrected for linoleic and linolenic acids, which are presumed to be inactive.

Because the efficiency of the liquid scintillation spectrometer is different for fatty acids and cholesterol, and also differs from time to time, the counts are calculated to 100% efficiency, or to distintegrations. The presence of dietary tallow results in a much reduced level of activity in both fatty acids and cholesterol. One might be tempted to explain the low activity levels in the fatty acids on dilution with deposited tallow acids. This cannot explain the low levels in the cholesterol, however. But if one assumes that the tallow in the diet results in a large acetate pool which dilutes the injected acetate, this could result in lowered levels of activity in both fatty acids and cholesterol.

The differences in the activities of the lipids on the trilinolein and trilinolenin diets, or between the fatty acids and cholesterol, are not obvious and are probably the resultant of a number of factors. It remains for future work to resolve these factors.

TABLE 7

Activity of egg fatty acids and cholesterol[1]

Dietary lipids	Active fatty acids[2]	Cholesterol
	dis./min./mg	*dis./min./mg*
Trilinolein, 5%	2470	1379
Trilinolenin, 5%	1164	1483
Trilinolein, 4.85% + tallow, 5.15%	586	462
Trilinolenin, 5% + tallow, 5%	727	657

[1] Activities were corrected to disintegrations/milligram/minute because of changes in the efficiency of the scintillation spectrometer.

[2] Values are corrected for linoleic and linolenic acids which are assumed to be inactive.

SUMMARY

1. The levels of linoleic and linolenic acid incorporation into the egg lipids increased with the amounts of their presence in the diet and reached plateaus at the 5% dietary level. Linoleic acid reached a higher level than linolenic acid. The levels of incorporation of each were decreased, however, when tallow was included in the ration.

2. Linoleic acid is the precursor of arachidonic and docosapentaenoic acids, whereas linolenic is the precursor of eicosapentaenoic and docosahexaenoic acids.

3. When linoleic acid was fed without tallow, there was a higher degree of incorporation of labeled acetate in the yolk fatty acids than when linolenic acid was fed, but the degree of its incorporation into cholesterol was the same with both. The inclusion of tallow in the diet markedly reduced the degree of incorporation of acetate into both fatty acids and cholesterol in which case the levels of incorporation into both acids and cholesterol were higher with linolenic acid ingestion.

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An Evaluation of the FAO Amino Acid Reference Pattern in Human Nutrition

I. STUDIES WITH YOUNG MEN[1]

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A study of the essential amino acids as an independent parameter of protein nutrition was made possible by the experiments of Rose ('49) identifying the 8 amino acids needed preformed in the diet of young adults for the maintenance of nitrogen equilibrium. The quantitative requirement for each of these amino acids has now been investigated under dietary conditions where the total nitrogen is maintained at a constant and adequate amount. These studies with both young men and women have been reviewed recently by Rose ('57) and by Leverton ('59). It has been suggested (FAO, '57) that the essential amino acid requirement values might serve as a basis for constructing a desirable reference pattern for the dietary amino acids.

The present report is an investigation of such an amino acid pattern derived from requirement values, the Food and Agriculture Organization (FAO) provisional pattern (FAO, '57). The amino acid pattern of whole egg was also studied together with modifications of FAO and egg patterns achieved by increasing or decreasing one or more of the essential amino acids. These various patterns were compared as to their effects on nitrogen equilibrium when they were administered to young male subjects in diets containing 10 gm of total nitrogen per day.

The results of this study add to the very limited information available on the minimal amount of total essential nitrogen required to maintain nitrogen balance when there is adequate nonessential nitrogen in the diet. In literature reports on experiments where these conditions prevailed, Rose and Wixom ('55) fed two young men purified essential amino acid mixtures containing 1.42 gm of nitrogen and observed them to be in nitrogen equilibrium over a wide range of total nitrogen intake. With whole egg as a source of essential amino acids (Swendseid et al., '59) three young men showed requirements ranging up to 0.9 gm of essential nitrogen when the total nitrogen intake was 6.5 gm.

EXPERIMENTAL PROCEDURES

Subjects were 12 male college students in good health as determined by a medical examination. Their ages ranged from 20 to 26 years and their weights from 60.5 to 87.3 kg. They were fed the various amino acid patterns, in controlled diets, and their response was measured using the nitrogen balance technique. The experimental methods have been described previously (Swendseid et al., '59).

The subjects received first a controlled diet of ordinary food containing 10 gm of nitrogen per day with calories adjusted to need. This diet was continued for 7 days to bring the subjects into nitrogen equilibrium on a constant nitrogen intake.

When the amino acid patterns were investigated, the experimental diet was administered in an amount that was isonitrogenous with the ordinary food diet. The FAO pattern was supplied in certain die-

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tary periods by purified amino acids and in others by whole egg plus the relatively small amounts of purified amino acids required to complete the FAO pattern. The egg pattern was also provided in certain dietary periods by purified amino acids and in others by the intact proteins in whole egg. Hence, in addition to comparing the effects of the two patterns, the effects of feeding amino acid mixtures and intact protein could also be compared. The purified amino acids used in this study were the L-isomeric forms in all instances and they were shown by analysis to be at least 99% pure.[3] To prepare the eggs, fresh eggs were hard-cooked and put twice through a Foley sieve.

Components of the diet other than purified essential amino acids (PEAA) and whole egg were as follows: (1) some ordinary food low in protein, referred to as the basal diet; (2) with the exception of glycine, a constant amount of purified nonessential amino acids (PNEAA) in the proportions in which they occur in whole egg; (3) variable amounts of glycine and diammonium citrate as needed to adjust the total nitrogen intake to 10 gm per day; and (4) variable amounts of nitrogen-free foods to adjust the caloric intakes to 50 to 55 Cal. per kg of body weight per day. The proteins of the basal diet were supplemented with PEAA so that the pattern under study was maintained in the entire diet.

In the first experiment, the amount of each amino acid that was fed for both patterns was proportioned to a selected amount of tryptophan, the amino acid assigned a value of unity in expressing the amino acid ratios of FAO and egg patterns (table 1). The amounts of tryptophan which were administered during the course of the study ranged from 240 to 440 mg per day with other amino acids being supplied in amounts proportioned to the pattern under investigation. The daily intakes of amino acids for representative diets, namely, those containing 360 mg of tryptophan but having different patterns of essential amino acids are shown in table 1.

The distribution of nitrogen for representative diets is shown in table 2. These diets contain 360 mg of tryptophan with other essential amino acids in amounts proportioned to egg pattern when the chief source of the essential amino acids is a PEAA mixture and when the chief source is intact protein from whole egg. For diets where the essential amino acids are proportioned to the reference pattern, it can be calculated that the amount of PEAA mixture and whole egg fed would be less with a given tryptophan intake than for egg pattern (table 1) and therefore the amount of glycine and diammonium citrate would be increased slightly to maintain a 10-gm nitrogen intake.

[3] We wish to express our appreciation to Dr. M. S. Dunn of U.C.L.A. for the determinations on the purity of the L-amino acids used in this study. The amino acids were purchased from Nutritional Biochemicals Corporation, Cleveland, and the H. M. Chemical Company, Ltd., Santa Monica, California.

TABLE 1

Ratios and daily intakes of essential amino acids using FAO and egg patterns

L-Amino acid	Pattern ratios, tryptophan = 1		Daily intake, tryptophan = 0.36 gm		Tentative minimum requirements of Rose
	FAO	Egg	FAO pattern	Egg pattern	
			gm/day	*gm/day*	*gm/day*
Tryptophan	1.0	1.0	0.36	0.36	0.25
Isoleucine	3.0	3.7	1.08	1.33	0.70
Leucine	3.4	5.4	1.22	1.93	1.10
Lysine	3.0	5.4	1.08	1.63	0.30
Methionine and cystine	3.0	3.7	1.08	1.32	1.10
Phenylalanine and tyrosine	4.0	4.7	1.44	1.67	1.10
Threonine	2.0	3.1	0.72	1.10	0.50
Valine	3.0	3.9	1.08	1.30	0.80
Total essential amino acid nitrogen			0.95	1.27	0.73

TABLE 2

Sources of dietary nitrogen with total N intake of 10 gm

Component	Diet containing purified essential amino acids			Diet containing whole egg		
	Amount[1]	Total N content	EAAN[2] content	Amount[1]	Total N content	EAAN[2] content
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Basal diet[3]	680.0	0.64	0.09	680.0	0.64	0.09
Glycine	18.8	3.51	—	17.1	3.19	—
Diammonium citrate	28.2	3.51	—	25.7	3.19	—
Whole fresh egg	—	—	—	147.9	2.96	1.15
Purified essential amino acids	10.3	1.18	1.18	0.3	0.03	0.03
Purified nonessential amino acids[3]	8.2	1.16	—	—	—	—

[1] Amount varies except for basal diet. Values shown are for a diet containing 360 mg of tryptophan with other essential amino acids in amounts proportioned to egg pattern.

[2] EAAN indicates essential amino acid nitrogen.

[3] We are indebted to Dr. M. J. Horn, Human Nutrition Research Division, U. S. Department of Agriculture for the amino acid analysis of the proteins in the basal diet and the nonessential amino acid analysis of whole egg.

In addition to obtaining data evaluating FAO and egg patterns on the basis of tryptophan equivalents, studies were made wherein the patterns were compared when the diets were isonitrogenous with respect to the amount of essential nitrogen. In these experiments with isonitrogenous amounts of the amino acid patterns, the chief source of the essential amino acids was always the intact protein of whole egg.

From the results of these studies, it appeared desirable to test some modifications of egg and FAO patterns. These modifications involved either increasing or decreasing one or more of the essential amino acids while keeping the total nitrogen intake constant. Again the chief source of essential amino acids in the diet was whole egg.

RESULTS AND DISCUSSION

In table 3 are shown the nitrogen balances of young men fed the FAO and egg pattern mixtures in quantities which furnished tryptophan, the limiting amino acid of the egg pattern as compared with the FAO pattern (table 1) in amounts ranging from 240 to 440 mg per day with other amino acids in amounts proportioned to the pattern under study. The average daily nitrogen balance values represent periods which varied from 5 to 8 days depending on the constancy of the daily nitrogen urinary values. One subject, JS, appeared to have a caloric insufficiency when receiving the PEAA mixture since his nitrogen balance values showed no increase in retention when the amounts of essential amino acids fed were increased.

The large numbers of extremely negative nitrogen values which appear in the table were unexpected results. Many of these values were obtained when mixtures were fed that contained amino acids in amounts approximating or exceeding the previously determined minimal requirements for the individual amino acids (Rose, '57) (table 1). Apparently then, when all of the essential amino acids are fed in amounts approximating their minimal requirements, the resultant mixture is inadequate to maintain nitrogen equilibrium.

When the purified amino acid mixture was the chief source of essential amino acids for the FAO pattern (table 3), subject KC was not tested above the 280-mg tryptophan level where he was in negative balance and subject EH was in positive nitrogen balance when fed at a level of 440 mg of tryptophan. The other 4 subjects required a larger quantity of essential amino acids for maintaining nitrogen equilibrium than the mixture containing the essential amino acids in amounts proportioned to 440 mg of tryptophan.

When the mixture of purified essential amino acids in egg pattern proportions provided the same amount of tryptophan as furnished by the FAO reference pattern in a previous period, all subjects showed

TABLE 3

Nitrogen balances of young men fed according to FAO and egg patterns

Subject	Body weight	Tryptophan intake[1]	Average daily nitrogen balance values[3] (gm/day)			
			PEAA[2] mixtures		Whole egg	
			FAO pattern	Egg pattern	FAO pattern	Egg pattern
	kg	*mg/day*				
KC	74.6	240	−0.79(1)	−0.29(5)		−0.45(2)
		280	−0.28(3)		−0.11(4)	
TS	78.6	240	−2.38(1)			
		280	−1.35(2)			
		320	−1.03(3)	−0.39(5)	−0.48(6)	+0.40(4)
		440	−0.49(7)			
RD	75.5	240	−1.06(1)			−0.32(2)
		280	−0.53(3)			−0.50(4)
		320				−0.25(5)
		440	−0.45(1b)	+0.14(3b)	+0.01(4b)	+0.64(2b)
EH	79.1	360	−0.50(1)	+1.48(4)	+0.21(2b)	+1.41(3)
		440	+0.08(2)		+0.55(1b)	
MM	68.2	360	−0.91(1)	+0.78(2b)		+0.62(3)
		440	−0.34(2)		+0.25(1b)	
JS	87.3	360	−0.35(1)	+0.03(3)		+0.04(2)
			−0.96(1b)			+0.08(4b)
		440	−1.02(2b)		−0.32(3b)	

[1] Other essential amino acids in amounts proportioned to pattern under study.

[2] PEAA indicates purified essential amino acid.

[3] The dietary periods varied in length from 5 to 8 days. Figures in parentheses show the sequence of dietary periods. The notation "b" refers to the fact that it is the second experimental study period for this subject.

either a distinctly less negative nitrogen balance or a positive balance. Hence for 6 subjects studied, better nitrogen balances were obtained when dietary essential amino acids were supplied in the egg pattern rather than in the FAO pattern proportions.

When whole fresh egg was substituted for purified amino acids as the chief source of essential amino acids in both patterns, the nitrogen balances of 5 subjects (TS, RD, EH, MM, and JS) were better when using the egg pattern than with the FAO pattern even though for two of the 5 subjects, MM and JS, the comparison was made with the FAO pattern based on a larger amount of tryptophan than the egg pattern.

The nitrogen balance values obtained in this study can also be used to compare the purified amino acid mixture with whole egg as the source of amino acids for a particular pattern. For the FAO pattern, excluding JS because of a probable caloric deficit in this period, 4 out of 5 subjects (TS, RD, EH, and MM) showed better nitrogen balances when amino acids were furnished by whole egg than when they were given as a PEAA mixture. For the egg pattern, two out of 6 subjects (TS and RD) showed better nitrogen balances when amino acids were furnished by whole egg than when they were given as a PEAA mixture; the other 4 subjects had essentially the same nitrogen balances regardless of the source of amino acids. Thus the data indicate that some subjects had better nitrogen balances when dietary amino acids were furnished by whole egg rather than by purified amino acids, but this was not a consistent finding. These results obtained with egg protein which has a high coefficient of digestibility (Sumner and Murlin, '38) do not extend necessarily to other food proteins, particularly those that are not completely digested (Allison, '60).

With respect to the more favorable nitrogen balances obtained with egg as compared with those obtained when using the FAO pattern, a possible explanation can be seen in table 1 where the daily intakes of

TABLE 4

Nitrogen balances of young men fed isonitrogenous amounts of amino acids in FAO and egg patterns

Subject	Body weight	FAO pattern			Egg pattern		
		Tryptophan intake[1]	EAA[2] nitrogen	Nitrogen balance[3]	Tryptophan intake[1]	EAA nitrogen	Nitrogen balance[3]
	kg	*mg*	*gm*	*gm/day*	*mg*	*gm*	*gm/day*
EH	79.1	320	0.84	−0.31	240	0.86	−0.20
MB	63.2	360	0.95	−0.04	280	1.00	−0.27
SK	60.5	360	0.95	−0.21	280	1.00	−0.26
TS	78.6	440	1.15	+0.09	360	1.27	+0.06

[1] Other essential amino acids as in pattern under study.
[2] EAA indicates essential amino acid.
[3] Average daily values of periods varying from 6 to 9 days.

each of the essential amino acids and the total essential nitrogen intake are shown when each pattern furnished 360 mg of tryptophan. It can be calculated that the egg pattern furnished 60% more of leucine than the FAO pattern, as well as 50% more of lysine and 20 to 30% more of the remaining essential amino acids with the exception of tryptophan. As a net effect there is a larger amount of total nitrogen from the essential amino acids, including cystine and tyrosine, for egg pattern, 1.27 gm as compared with 0.95 gm for the FAO pattern. This same situation can also occur in the comparison of food proteins of differing essential amino acid composition. Here too, a variation in the percentage composition of the essential amino acids can obscure the relationship between the essential amino acid patterns (Harper and Kumta, '59).

It seems, therefore, that in the evaluation of essential amino acid patterns, consideration should be given to studies wherein the total amount of essential amino acids or the total essential amino acid nitrogen supplied by the patterns is present in equal quantities. Such a comparison can be made for two subjects in table 3. Subject TS, when given PEAA mixtures, showed an average nitrogen balance value of − 0.49 gm with the FAO pattern as compared with − 0.39 gm with an isonitrogenous egg pattern. Subject RD, when supplied with whole egg protein, showed an average nitrogen balance value of + 0.01 gm for the FAO pattern as compared with − 0.25 gm for isonitrogenous egg pattern.

In table 4, additional nitrogen balance values are given for 4 subjects who were fed isonitrogenous amounts of amino acids according to FAO and egg patterns. Since previous results (table 3) showed no consistent variations in nitrogen balance values when PEAA mixtures were replaced by egg protein, it was decided in this study to use egg protein as the chief source of essential amino acids. The dietary periods were from 6 to 9 days in length. The nitrogen balance values shown in table 4 are either negative or very slightly positive and hence are in the region of nitrogen equilibrium where there is a linear relationship between nitrogen intake and nitrogen balance (Allison, '55). The nitrogen balance values for all 4 subjects are similar with FAO and egg patterns when these diets contained isonitrogenous amounts of essential amino acids. These data together with those of the two subjects, TS and RD, (table 3) indicate that under the experimental conditions tested here, there is no apparent difference in the metabolic response that can be distinguished on the basis of nitrogen balance values when amino acids in the FAO or egg patterns are administered in isonitrogenous amounts.

Howe et al. ('60), studying the growth rate of weanling rats and comparing the FAO reference amino acid standard with casein, observed a relatively lower nutritive value for the FAO mixture. When, however, the percentage of essential amino acids fed as FAO pattern was the same as casein, the FAO reference mixtures promoted somewhat greater growth than casein. These results and the findings of the

TABLE 5

Effect of various modifications of FAO and egg patterns on nitrogen balance

Subject	Body weight	Amino acid pattern	EAA nitrogen	Dietary modification	Nitrogen balance[1]
	kg		*gm*		*gm/day*
AS	70.5	FAO	0.95	None	−0.14
		FAO	0.95	Minus 80 mg tryptophan	+0.05
MB	63.2	FAO	0.95	None	−0.04
		FAO	0.95	Minus 80 mg tryptophan	+0.06
		FAO	0.95	Plus 100 mg leucine, 100 mg lysine, 100 mg threonine	−0.01
TS	78.6	FAO	0.95	None	−0.63
		FAO	0.95	Plus 100 mg leucine, 100 mg lysine, 100 mg threonine	−0.19
EH	79.1	FAO	0.83	None	−0.31
		FAO	0.83	Plus 100 mg leucine, 100 mg lysine, 100 mg threonine	−0.22
SK	60.5	FAO	0.95	None	−0.21
		FAO	0.95	Plus 100 mg leucine, 100 mg lysine, 100 mg threonine	−0.24
AH	78.6	Egg	1.00	None	+0.03
		Egg	1.00	Plus 200 mg phenylalanine	+0.11
HH	73.6	Egg	1.00	None	+0.07
		Egg	1.00	Plus 200 mg phenylalanine	−0.16
JS	87.3	Egg	1.00	None	+0.16
		Egg	1.00	Plus 200 mg phenylalanine	+0.20

[1] Average daily values of dietary periods varying from 6 to 9 days.

present study with humans emphasize the importance of maintaining equivalent percentages of essential amino acids or equal amounts of total nitrogen when comparing amino acid patterns.

When the amino acids in the FAO and egg patterns are fed in isonitrogenous amounts, the quantities of some individual amino acids vary in the two patterns. For example, tryptophan is higher in the FAO pattern than in the egg pattern and the aromatic amino acids are also slightly higher. Leucine, lysine and threonine are lower in the FAO than in egg pattern. The effect of the following modifications on nitrogen balance was therefore studied: (1) lowering the tryptophan in the FAO pattern; (2) increasing leucine, lysine and threonine in the FAO pattern; and (3) increasing phenylalanine in the egg pattern. The results are shown in table 5.

For subjects AS and MB, decreasing the amount of tryptophan by 80 mg had no effect on nitrogen balance when the diet contained the essential amino acids in FAO pattern proportions. This confirms the evidence obtained in the studies reported in table 4 and suggests that the tryptophan ratio in the FAO pattern is too high and that it can be lowered by at least 20%. Howe et al. ('60) reported also that the protein efficiency of the FAO pattern in promoting the growth of rats was not altered when the tryptophan content was decreased by 33 to 50%. The modified FAO pattern ratio resulting from a reduction in tryptophan content is very similar to the egg pattern.

Other results in table 5 indicate that with the exception of subject TS, who had a high requirement for essential amino acids, the addition of the leucine-lysine-threonine supplement had no appreciable effect on nitrogen balance. Hence no consistent results could be obtained which would indicate that any of these amino acids is limiting in the FAO pattern. Likewise from the experiments with phenyl-

alanine supplementation (subjects AH, HH, and JS), the lack of effect on nitrogen balance gives no evidence that this amino acid is limiting in egg protein. It is possible that the nitrogen balance method is not suitable for detecting limiting amino acids. Other criteria, such as changes in plasma amino acid concentrations (Longenecker and Hause, '59), may prove to be more sensitive indicators.

The results of this study also give information as to the total essential nitrogen requirements. When the criterion of adequacy is taken as a zero or slightly positive nitrogen balance value in all dietary periods investigated at a given essential nitrogen intake, then the approximate essential amino acid nitrogen requirements can be estimated for 7 of the 12 subjects studied in this series of experiments. Two subjects, AH and JS, had a requirement value of 1.00 gm of essential nitrogen per day; one subject, EH, a value of 1.15; 4 subjects, TS, RD, MM, and JS, a value of 1.27 (tables 3, 4, and 5). Four additional subjects were in nitrogen equilibrium during one test period when the total essential nitrogen was approximately 1.00 gm and thus would be expected to have requirements close to this value. Hence under the experimental conditions reported here, the total essential nitrogen requirements for these young men ranged from 1.00 to 1.30 gm per day. These values are greater than 0.73 gm of nitrogen per day which is the sum of the minimal requirements for the individual amino acids as determined by Rose ('57) (see table 1). In the present study, since all amino acids were fed in amounts approximating their minimal requirements, the total amount of essential nitrogen in the diet was considerably less than in previous studies (Rose, '57; Leverton, '59). It may be that when all of the essential amino acids are fed in amounts approaching their minimum, the requirement is increased. However, although these values were obtained when both the FAO reference and egg pattern mixtures were fed, there is the possibility that modifications of the patterns would result in a reduction of the total essential nitrogen requirements.

No correlation was found between body weight and total essential nitrogen requirements in the present experiment. For example, JS weighing 79.1 kg, had a total essential nitrogen requirement of 1.0 gm per day and MM, weighing 68.2 kg, had a requirement of 1.30 gm per day. This finding is in agreement with other reports on amino acid requirements studied in the presence of a constant total nitrogen intake (Rose, '57; Leverton, '59). However, Clark et al. ('60) have shown a correlation between body weight and the requirement for lysine in young men.

It is possible that results reported here for total essential nitrogen requirements and for comparisons of FAO and egg patterns might be altered by such factors as age, sex, the state of protein repletion and the source and amount of nonessential nitrogen. An investigation of essential amino acid patterns in young women using the experimental conditions of this study will be reported.

SUMMARY

Twelve young men were subjects for a study comparing the Food and Agriculture Organization (FAO) amino acid reference pattern and the amino acid pattern of egg as to their effects on nitrogen equilibrium at a constant total nitrogen intake of 10 gm per day.

In diet periods wherein the chief source of amino acids was a purified mixture, for both FAO and egg patterns, the nitrogen balance values were not consistently different from diet periods wherein the chief source of amino acids was egg.

When the diets were planned according to the FAO or egg patterns with the amino acids proportioned to an equal amount of tryptophan for both patterns, better nitrogen balance was obtained with egg pattern. When the diets contained isonitrogenous amounts of the amino acids according to the FAO or egg pattern, similar nitrogen balance values were found. Reducing the tryptophan content of the diets containing amino acids in FAO pattern proportions by approximately 20% had no effect on nitrogen equilibrium.

With either FAO or egg pattern, the total amount of essential nitrogen required in the diet to maintain nitrogen equilib-

rium in young men under the conditions of this study ranged from 1.0 to 1.3 gm per day.

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A Mechanism for the Copper-Molybdenum Interrelationship

III. REJECTION BY THE RAT OF MOLYBDATE-CONTAINING DIETS[1]

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An exceedingly complex family of syndromes has been attributed to the ingestion of toxic amounts of molybdenum. The degree of sensitivity to molybdenum and the nature of the symptoms encountered seem to reflect animal species differences, dietary levels of a number of inorganic materials such as copper, sulfate, zinc, and other metals, and dietary levels of such organic materials as protein, sulfur amino acids, and perhaps even vitamins. The complexity of this situation has been competently reviewed (Underwood, '56; Miller and Engel, '60).

Among the more common symptoms of molybdenum toxicity in higher animals is anorexia. Recent studies from this laboratory have demonstrated that anorexia may be not only a symptom of molybdenum toxicity, but the actual cause of metabolic alterations in the animal which contributed to the molybdenum-induced problems with copper metabolism (Siegel and Monty, '61).

The present endeavors demonstrate the ability of rats to voluntarily reject diets containing toxic amounts of molybdenum. It is suggested that this ability may reinforce a true loss of appetite in the animals, and serve as a mechanism for the animal to limit intake of a toxic dietary constituent. Sensory recognition of the presence of molybdenum is instrumental in the process of voluntary rejection.

EXPERIMENTAL

Young male albino rats of the Wistar strain[2] were used in all experiments. The body weight of individuals was within the span of 65 to 75 gm at the start of each experiment.

The basal diet used was constituted from vitamin-free casein,[3] glucose,[4] corn oil,[5] salts and vitamins as described earlier (Halverson et al., '60). Modifications of the basal diet consisted of the addition of either or both of the following: 800 ppm of molybdenum as sodium molybdate; and 0.29% of sulfate as sodium sulfate. The experimental precautions for the use of low-copper diets (Halverson et al., '60) were observed, including the administration of distilled water, although no significance is ascribed to this treatment in the present endeavors.

Cages were of stainless steel construction, individually suspended from above, having solid walls but wire-mesh floor and front. Dimensions were 10″ long, 7″ wide and 7″ high. Food containers were clear glass cylinders, 3″ in diameter and 2″ deep, equipped with white-lacquered screw-cap metal lids having a 1¼″ hole stamped through the middle to minimize spillage of the food.

In experiments involving food selection, the two food containers were placed in the forward corners of the cage, with the water drinking tube between them. Care was taken to make all food containers identical in appearance except for the identification number appearing on the bottom of each one. The position of the food containers

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[2] Obtained from Albino Farms, Red Bank, New Jersey.

[3] Obtained from Nutritional Biochemicals Corp.

[4] USP grade, obtained from Merck and Company, Rahway, New Jersey.

[5] Mazola, Corn Products Company, New York.

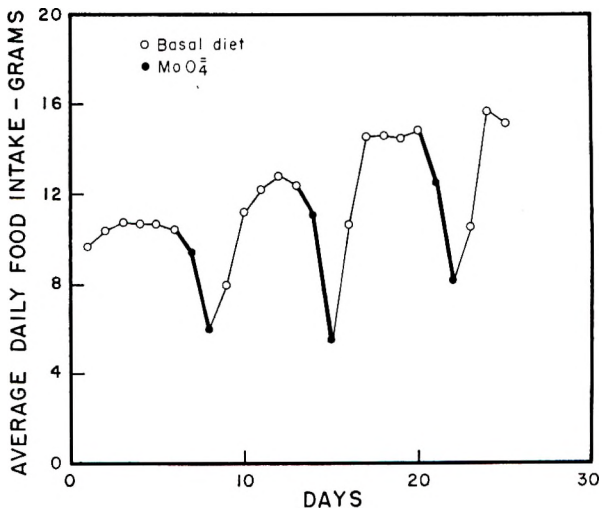

Fig. 1 Effect of molybdate on food intake. Molybdate included at 800 ppm Mo. Data represent averages obtained with 20 animals.

was alternated according to a prearranged schedule in these experiments. Also, the containers were completely emptied and the contents replaced on a schedule, and the containers replaced with clean ones on another schedule.

Food consumed from each container was measured daily, and the contents of the pair of food dishes in each cage replenished to equal levels. For each cage containing one or two animals the percentage of the total daily food intake consumed from each container was recorded. Group statistics were obtained as the average of these individual daily percentages for the number of animals in the experiment.

RESULTS

The introduction of molybdate to the diet elicited a prompt drop in food intake to a new level which is established within the first 24 hours. Replacing the molybdate-containing diet with a control diet produced a rapid recovery to "normal" levels of food intake. In figure 1 is presented a demonstration of the response of food intake to cyclic short-term challenges with a molybdate-containing diet.

The gradual expression of a discrimination against the toxic molybdenum diet by animals offered a choice between a basal diet and one containing 800 ppm of molybdenum is demonstrated in figure 2. After 35 days the animals were about 70% successful in rejecting the molybdate-containing diet. Also demonstrated in figure 2 is the measurable effect of changing food containers or replacing their contents. Each such operation produced a minor fluctuation in the food selection curves, usually with the result of reducing the degree of discrimination.

The toxic properties of the 800 ppm molybdenum diet used in these studies may be alleviated by the inclusion of sulfate at a concentration of 0.29% (Mills et al., '58; Halverson et al., '60). When animals were offered a choice between diets containing sulfate alone or sulfate and molybdate, discrimination against the molybdate-containing diet was not expressed during the 35 days of the experiment (fig. 3). Two possible explanations of this phenomenon were apparent. Either the animals were not induced to discriminate against a nontoxic molybdate-containing diet, or the presence of sulfate prevented the recognition of the presence of molybdate.

Evidence against the latter possibility is presented in figure 4; animals failed to discriminate between a toxic molybdate-containing diet and a second diet rendered nontoxic by the inclusion of sulfate. Had the presence of sulfate obscured the properties of the diet relied upon for discrimination against molybdate in experiments like that of figure 2, it should have been

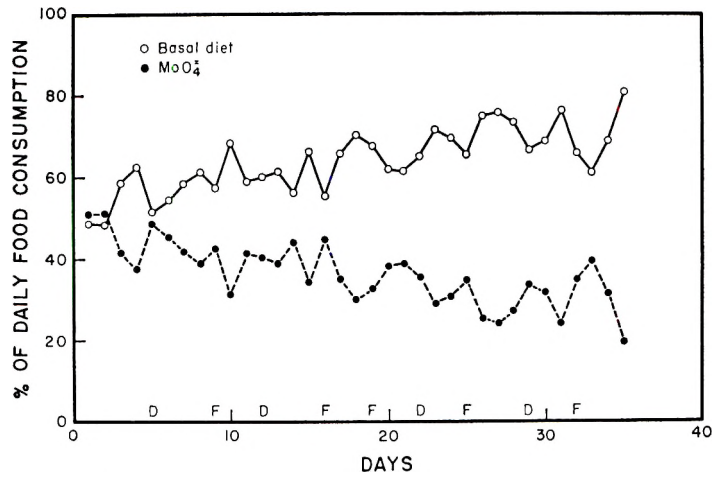

Fig. 2 Selection between a basal diet and one containing molybdate. Molybdate included at 800 ppm Mo. D denotes replacement of food containers; F, replacement of containers' contents. The data immediately above each symbol indicate the results for the 24-hour period following the operation. Position of food containers alternated daily. Data represent averages obtained with 20 animals.

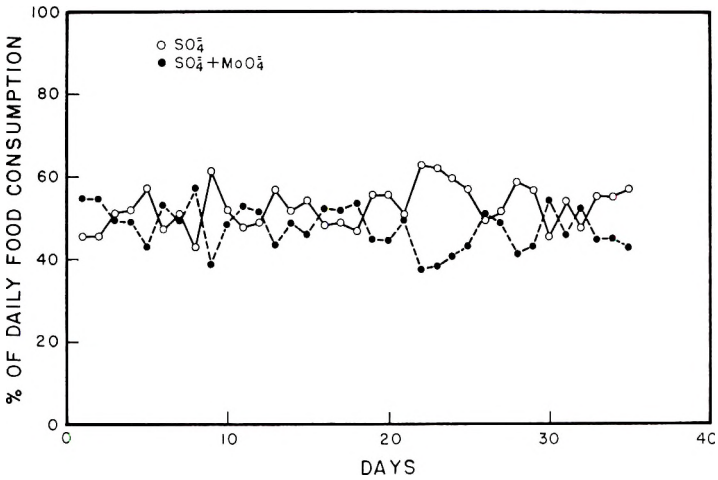

Fig. 3 Selection between diets containing sulfate alone or sulfate and molybdate. Sulfate included at 0.29% level, molybdate at 800 ppm Mo. Position of food containers was alternated daily. Data represent averages obtained with 10 animals.

possible for the animals to reject the toxic diet containing molybdate alone in the experiment of figure 4.

In the experiments just described, the position of the two food containers in each cage was alternated daily. By limiting the alternation of position to every third day the development of discrimination against molybdate in a choice between basal diet and one containing a toxic level of molybdate can be greatly accelerated. The three-day alternation cycle permitted the achievement of 70% discrimination in 10 to 14 days (fig. 5). Twice this period of time transpired in the achievement of the same level of discrimination with daily alternation of the position of food containers (fig. 2).

Considerable individual variability in the capacity to develop discrimination against the molybdate-containing diet was noted among our experimental animals.

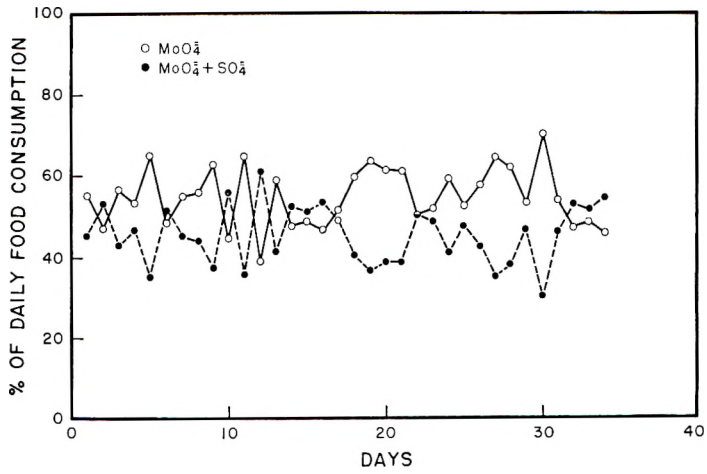

Fig. 4 Selection between diets containing molydate alone or sulfate and molybdate. Sulfate included at 0.29% level, molybdate at 800 ppm Mo. Position of food containers alternated daily. Data represent averages obtained with 10 animals initially gradually diminishing to 6 survivors.

By removing from a group the individuals exhibiting less reliable discrimination against molybdate, it was possible to demonstrate that the recognition of the presence of molybdate requires some "aging" of the diet to which molybdate has been added. In figure 6 is illustrated the discrimination against molybdate by 12 selected members of the group shown in figure 5. In the first 14 days, an average discrimination level of better than 80% was obtained, whereas individuals within this sub-group demonstrated reliable discrimination levels of better than 95%. On the 15th and 24th days, the animals were supplied with freshly prepared diets, constituted on the day they were first offered to the animals. A temporary loss of discrimination against molybdate occurred in each case. This phenomenon has been observed repeatedly with three different groups of selected animals, challenged with freshly prepared diets as many as 5 successive times. The magnitude and duration of the effect, however, vary considerably. Diets which had been aged for at least 5 days (at 40°C) could be offered to such groups of animals without disturbing their pre-established level of discrimination against molybdate.

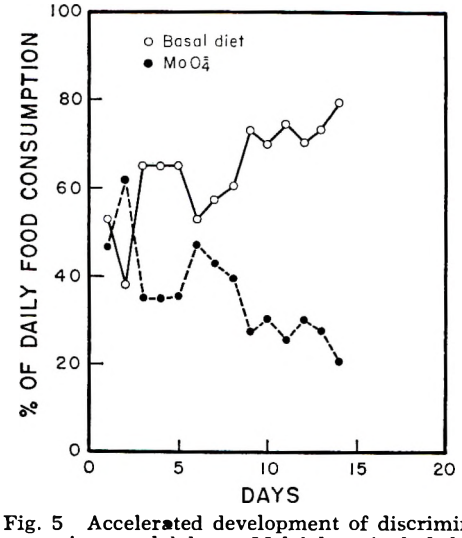

Fig. 5 Accelerated development of discrimination against molybdate. Molybdate included at 800 ppm Mo. Position of food containers alternated every third day. Data represent averages obtained with 19 animals.

DISCUSSION

The development of the ability to discriminate against a toxic molybdate-containing diet requires a learning or conditioning period. Factors recognized to be important to the duration of the conditioning period include the frequency of alternation of the position of food containers and the frequency of replacement of the contents of the containers. It is probable that this conditioned recognition of molybdate diets has its foundation in the development by the animals of a correlation

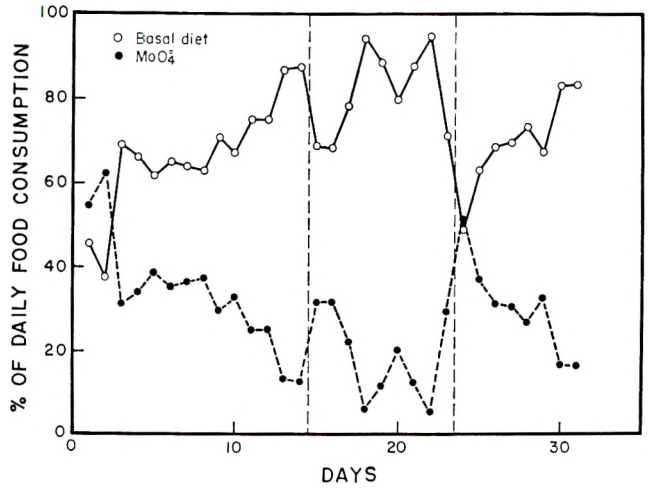

Fig. 6 Effect of freshly prepared diets on the discrimination against molybdate. Molybdate included at 800 ppm Mo. Position of food containers alternated every third day. Introduction of fresh diets on days 15 and 24. Data represent averages obtained with 12 animals.

between a physiological response to the ingestion of toxic amounts of molybdate and a sensory attribute (namely, taste and odor) of the presence of molybdate in the diet. Several analogies to such a situation may be found in the classic work of Richter and his associates (Richter, '43; Richter and Clisby, '41).

This viewpoint can be supported by a number of experimental observations. It is significant that molybdate-containing diets are not rejected per se. This fact is demonstrated by the failure to obtain discrimination against a nontoxic diet containing molybdate in the choice between diets supplemented with molybdate and sulfate or with sulfate alone (fig. 3). Additional proof of this point was obtained in an experiment (not illustrated) wherein animals previously conditioned against molybdate, in a choice of basal and molybdate diets, were offered a choice between diets containing molybdate and sulfate or sulfate alone. Discrimination against the nontoxic molybdate diet was apparent for several days and then gradually subsided. The implication is that when the toxicity of molybdate was alleviated, the motivation for sensory discrimination against molybdate declined. This behavior could be referred to as deconditioning.

Further evidence for the argument would include the fact that a toxic diet was not differentiated from a nontoxic diet when both contained molybdate (fig. 4). In addition, it was a common observation in these experiments that animals conditioned against molybdate would reject the molybdate-containing diet without apparently tasting it. This phenomenon would certainly implicate sensory recognition, probably olfactory, of the presence of molybdate, and would explain how some animals could reliably maintain discrimination levels of better than 95%, as mentioned earlier.

The sensory attribute relied upon in discrimination against molybdate apparently is lacking or insufficiently developed in freshly prepared diets. The characteristic odor of the molybdate-containing diets, therefore, is probably developed by an interaction between molybdate and dietary constituents. It would be unexpected, indeed, if molybdate itself could be detected in these diets, since it is nonvolatile and represents as a salt only 0.2% of a diet which is otherwise 6% salts by composition.

No insight into the nature of the physiological response which motivates the animal to discriminate against toxic molybdate-containing diets has been obtained in this study. It may be significant that diarrhea is a common symptom of molybdenum toxicity with the diet used in the present investigations (Halverson et al., '60), and that sulfate alleviates this part

of the syndrome. Thus, disturbances of the gastrointestinal system may follow promptly upon the ingestion of molybdate and serve as the motivating physiological condition.

With the recognition that bacterial sulfur metabolism may be inhibited by molybdate,[6] the possibility exists that disturbances of the gastrointestinal tract result from molybdenum-induced alterations of the microbiological population of the intestinal lumen. Attempts to evaluate the significance of molybdenum-flora interactions in the dietary interrelationship of molybdenum, copper and sulfur in the rat are currently underway. It is readily anticipated that similar effects upon the population of the rumen would have great impact upon the nutrition and metabolism of polygastric animals.

SUMMARY

Rats evince the ability to develop a sensory recognition of the presence of molybdenum in the diet, and to make use of this ability in the efficient rejection of diets containing toxic levels of that metal. The sensory factor relied upon appears to result from the interaction of molybdate with other dietary constituents. Factors operative in the development of discrimination against molybdenum are delineated and discussed.

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Increased Lipotropic Requirements with Renal Necrosis Induced in Rats by High-Fat Diets[1,2]

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Over 20 years ago Griffith and Wade ('39) demonstrated that choline was essential to the survival of weanling rats. Since that time, numerous studies have clearly defined the morphologic changes at death, principally the hemorrhagic renal necrosis, in such animals deprived of choline (Griffith and Nyc, '54). This renal necrosis, occurring after approximately one week, is usually preceded by the development of a fatty liver and is obtained only with severe dietary deficiencies of lipotropic factors.

The accumulation of fat in livers, kidneys and hearts (Wilgram et al., '54) of choline-deficient animals has stimulated the study of the effects of various dietary fats on choline requirement. Hartroft ('55) has reviewed these studies, which in general indicate an exaggeration of choline deficiency by fats containing a large proportion of long-chain saturated fatty acids.

In the course of studies of high-fat diets in rats, principally in relation to cardiovascular disease (Thomas et al., '59), we have found a striking effect of certain dietary fats on the choline requirement of weanling rats. Results of experiments pertaining to this effect are reported herein.

MATERIALS AND METHODS

Food was prepared in quantities sufficient for the entire experimental period. Dry ingredients were first mixed well, and then melted fat added during stirring with a Hobart food mixer. The food was kept refrigerated and weighed amounts, in excess of consumption, were offered at two-day intervals.

Composition of the "basal diet," which produced a high incidence of renal necrosis, is shown in table 1. This diet and its variations were fed to 280 weanling rats in 6 different experiments composed of 21 separate dietary groups (table 2). All rats were male Wistar albinos obtained from Charles River Breeding Laboratories.[3] The animals were kept in individual wire-bottom cages in an air-conditioned animal room and given water and diet ad libitum. They were weighed weekly. Autopsies were performed on every animal that died during the experiments and all survivors were killed and autopsied after an experimental period of two weeks except in experiment 1, in which only the animals dying during the first two weeks were autopsied. Histologic sections of heart, liver, and kidneys

TABLE 1
Basal diet for renal necrosis

	% by weight
Casein	20
Cocoa butter	40
Cholesterol	5
Salt mixture[1]	4
Vitamin mixture[2]	2
Non-nutritive cellulose[3]	5
Sucrose	24

[1] Wesson, ('32).

[2] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, supplying 0.16% of choline in the diet. Each kilogram of the vitamin mixture contained the following, triturated in dextrose: (in grams) vitamin A concentrate (200,000 units/gm), 4.5; vitamin D concentrate (400,000 units/gm), 0.25; α-tocopherol, 5; ascorbic acid, 45; inositol, 5; menadione, 2.25; *p*-aminobenzoic acid, 5; niacin, 4.5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 1; Ca pantothenate, 3; biotin, 0.02; and folic acid, 0.09.

[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

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[2] Supported by U.S.P.H.S. research grants H-4089 and H-3525 from the National Heart Institute, Bethesda, Maryland.

[3] Cambridge, Massachusetts.

TABLE 2

Experimental data concerning renal necrosis in rats fed variations of the high-fat diet

Experiment	Group	No. of rats	Starting weight	% with necrosis	Dietary fat % by weight	Other dietary variation[1]
			gm			
1	1	30	43	60	Cocoa butter, 40%	(Basal diet)
	2	60	44	10	Cocoa butter, 40%	Na cholate, 2%
2	3	10	49	50	Cocoa butter, 40%	(Basal diet)
	4	10	49	0	Cocoa butter, 50%	No cholesterol
	5	10	49	0	Cocoa butter, 40%	Choline chloride, 1%
	6	10	49	40	Cocoa butter, 35%	Corn oil, 5%
	7	10	49	30	Cocoa butter, 40%	Vitamin E by dropper daily
3	8	10	42	80	Cocoa butter, 40%	(Basal diet)
	9	10	43	70	Cocoa butter, 40%	No pyridoxine
	10	10	41	0	Cocoa butter, 40%	Choline chloride, 1%
4	11	10	58	90	Cocoa butter, 40%	(Basal diet)
	12	10	58	10	Cocoa butter, 40%	No cholesterol
	13	10	59	0	Butter (dairy), 40%	
	14	10	58	40	Corn oil, 40%	
	15	10	58	20	Cocoa butter, 40%	Na cholate, 2%
	16	10	58	0	Cocoa butter, 40%	Choline chloride, 1%
5	17	10	45	50	Cocoa butter, 40%	(Basal diet)
	18	10	45	0	Cocoa butter, 2%	
	19	10	45	0	Butter (dairy), 2%	
	20	10	45	0	Cocoa butter, 20%	
	21	10	45	0	Butter (dairy), 20%	

[1] All changes in percentage composition of the diet shown in table 1 were compensated for by increasing or decreasing the percentage of dietary sucrose unless shown above.

of all rats in experiments 2 and 3 and of selected rats in other groups were prepared by fixing in 4% formaldehyde, embedding in polyethylene glycol[4] (Jones et al., '59) and staining with hematoxylin and eosin, periodic acid-Schiff (PAS) and with oil red O for lipid. Selected blocks of kidney were also fixed in 1% osmium tetroxide, embedded in methacrylate, sectioned on an ultra-microtome and examined with an RCA-EMU3C electron microscope.

RESULTS

Results of the experiments are shown in figure 1 and table 2 and partially summarized in table 3. In the groups fed the basal diet, 30 of the 70 rats died during the two weeks' period, all 30 having evidence of advanced hemorrhagic renal necrosis at autopsy, and 14 more had gross or microscopic evidence of renal necrosis or both when killed at two weeks. The disease was morphologically similar in all groups in which it occurred. The kidneys were swollen and the cortices, as seen on the surface and on cut section, were mottled yellow and red. In the animals that were killed, the swollen kidneys were not always hemorrhagic, but were sometimes pale and flecked with yellow, probably representing an early stage of the disease.

Microscopically, the most prominent change was necrosis of tubular epithelium, the proximal convoluted tubules being most severely affected, with pyknosis and loss of nuclei in the epithelial cells (fig. 2). Casts were present in the descending portion of the proximal convoluted tubule (fig. 3). In addition, it was common to find hemorrhage into interstitial tissue of the cortex (fig. 4) and renal capsule (fig. 5) as well as foci of calcification in the necrotic areas. Intensely PAS-positive, eosinophilic casts filled the collecting tubules (fig. 6) even in early stages of necrosis, a feature that has not been emphasized in previous reports. Oil red O stains revealed stainable lipid in the tubular epithelial cells, basilar in location in non-necrotic cells (fig. 7) and distributed widely within and without cells in the necrotic areas.

[4] Carbowax, Union Carbide Chemical Company, Charleston, West Virginia.

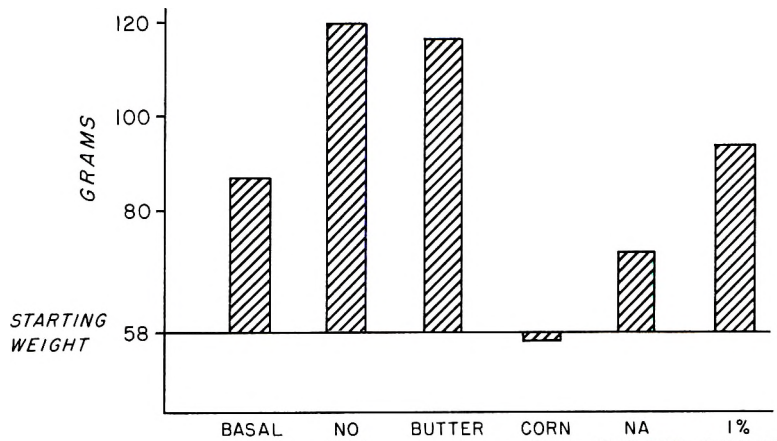

Fig. 1 Average body weights of 6 experimental groups (exp. 4, table 2) at the end of the two-weeks' experimental period. The food intake in average grams per day and the percentage of animals developing renal necrosis are shown below the bar for each group. Notice that there is no correlation between the percentage with necrosis and either food intake or growth.

TABLE 3

Summary—composite of data from table 2 with emphasis on dietary fat

No. of rats	Diet	% with necrosis
70	Cocoa butter, 40%, cholesterol 5%	65
20	Cocoa butter, 40%, no cholesterol	5
70	Cocoa butter, 40%, cholesterol, 5%, Na cholate, 2%	11
40	Cocoa butter, 40%, cholesterol, 5%, 1% choline added	0
10	Cocoa butter, 20%, cholesterol, 5%	0
10	Cocoa butter, 2% cholesterol, 5%	
10	Corn oil, 40%, cholesterol, 5%	40
10	Corn oil, 5% and cocoa butter, 35%, cholesterol, 5%	40
10	Butter, 40%, cholesterol, 5%	0
10	Butter, 2%, cholesterol, 5%	0

The presence of lipid in the basilar (below the nucleus) portion of epithelial cells was not confined to animals that developed necrosis, but was noted occasionally even in the rats given a supplement of 1% choline chloride. Electron microscopy was not helpful in the areas of advanced necrosis, but in adjacent areas the tubular epithelial cells showed two types of change: basal fat (fig. 8) as was seen in light microscopy, and dilatation of ergastoplasm in the supra-nuclear cytoplasm, creating a vacuolated appearance (fig. 9).

The livers of rats were grossly somewhat pale but lacked the marked yellow appearance characteristic of the fatty livers usually seen in adult rats fed a standard choline-deficient diet (Wilgram et al., '54). Microscopically, a moderate accumulation of fat was present in parenchymal cells, especially in pericentral areas, in the animals of groups receiving high-fat diets plus cholesterol. Within groups, no relationship was noted between the degree of fatty change in the liver and the presence of necrosis of the kidneys. Fatty cysts, as described by Hartroft ('50), were frequently present in the livers of rats in groups that had a high incidence of renal necrosis, but were never prominent.

The hearts of rats had occasional foci of myocardial necrosis and calcification (fig. 10) known to be associated with choline deficiency (Wilgram et al., '54; Keston et al., '45). This lesion was confined to groups in which renal necrosis occurred and, except for rare small foci, was present only in the animals with advanced renal disease.

Adrenals of rats that developed renal necrosis were significantly larger than those of rats not developing the disease (average 52 vs. 37 mg; $P < 0.001$). Microscopic examination of the enlarged adrenals revealed no increase in width of zona glomerulosa, suggesting that the increase was in the fascicular zone.

DISCUSSION

Results of the experiment indicate that high-fat diets markedly increase the requirement of weanling rats for choline. This increase is most strikingly evident when the dietary fat is cocoa butter. It is well known that a decrease in food intake of the rat is accompanied by a decrease in requirement for lipotropic factors but this factor cannot account for our results (fig. 1). Dietary intake and weight gains were similar in basal and choline-supplemented groups. Intake was relatively high and weight gain great in the group receiving butter, but rats in this group did not develop evidence of choline deficiency. Weight gain correlated moderately well with food intake except in the groups fed corn oil and sodium cholate (fig. 1), the dietary intake being lower but weight gain greater in the group fed sodium cholate and 40% cocoa butter than in the group fed 40% of corn oil. Sodium cholate may decrease the palatability of the diet and yet enhance absorption of fat.

Bile salt, in the form of sodium cholate, decreases the effect of cocoa butter on increasing choline requirement (groups 2 and 15 compared with 1 and 11, table 2). This action of sodium cholate may be the result of its emulsification of fat, with generally better intestinal function and more complete absorption of choline (0.16% in this diet) and protein. Other explanations could also explain this effect of sodium cholate. It has been postulated that one function of choline is concerned with the absorption of fat (Frazer, '46; Tidwell, '50), although some aspects of this have not been confirmed (Tasker and Hartroft, '49). Also, the role of choline in intravascular transport of fat has long been suspected (Best et al., '34). The possibility exists that sodium cholate may substitute to some degree for choline in one of these ways, thereby reducing the total requirement for lipotropic substances. These possibilities suggest future investigations.

Corn oil, substituted for cocoa butter in the "basal" renal-necrosis diet, was almost as effective as cocoa butter in the production of kidney disease, indicating that in this experimental situation the effect of a fat is not proportional to its content of C-14 to C-18 saturated fatty acids as suggested by Channon et al. ('42). These studies of Channon et al., however, were concerned with dietary fatty livers rather than renal necrosis and the dietary conditions were completely different from ours.

The basal diet used in our experiment contained 5% of cholesterol, a substance long known to increase choline requirement for prevention of fatty liver (Best et al., '34). Our studies indicate that cholesterol may similarly increase choline requirement for maintenance of healthy kidneys as well as livers. The high incidence of necrosis of kidneys obtained with the basal diet was obviously dependent on both the cholesterol and cocoa butter. It appears that weanling rats fed a relatively complete diet, but containing in addition 5% of cholesterol, will serve as a sensitive indicator for the effect of added dietary fat. This finding is particularly important in view of the use of high-fat, high-cholesterol diets in studies regarding experimental atherosclerosis. Such diets must therefore be formulated and results interpreted with the realization that a choline-deficiency state could be produced inadvertently by these means, even though choline and protein are present in amounts that are usually considered adequate.

We have no explanation for the complete protection provided, under the same dietary conditions, by dairy butter, as compared with either cocoa butter or corn oil. The extremely small choline content of dairy butter, as given by Engel ('43), is insignificant when compared with the

amount of choline (0.16%) already present in our basal diet. The protein content of butter is only 0.6%, and when this fat is fed at a 40% level, obviously is not a significant protein addition to a diet already containing 20% of casein.

This basal diet supplied 500 mg of methionine (in the casein) and 0.16 gm of choline (in the vitamin mixture) per 100 gm of diet. It was completely unexpected to find that these young animals (approximately 40- to 60-gm) developed hemorrhagic renal damage because production of this lesion has always been regarded as a manifestation of only the most severely hypolipotropic diets. But our basal diet already had a lipotropic content regarded as adequate for the weanling rat under most circumstances. The fact that the lipotropic requirement can be sufficiently increased as to result in such dramatic manifestations of choline deficiency raises the possibility that similar increases in demands for choline may have gone unrecognized in unusual circumstances in both animals and man, particularly in early life. The demonstration of an adequate amount of dietary lipotropic factors by normal standards does not rule out the above possibility, as shown in these experiments.

SUMMARY

In weanling rats a high-cholesterol diet provides a sensitive indicator for the effect of various dietary fats on choline requirement.

Our results indicate that under these conditions cocoa butter increased choline requirement markedly, whereas butter did not. This effect was not the result of saturation of fat, as corn oil is almost as effective as cocoa butter. A lipotropic effect of sodium cholate was observed in these experiments.

High-fat and high-cholesterol diets used in other experimental studies must be formulated with care because even with usually adequate amounts of choline, a deficiency state can be induced.

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PLATE 1

EXPLANATION OF FIGURES

2 Renal cortex of a rat with advanced necrosis. Tubular epithelial cells have completely lost their nuclei. H & E. × 540.

3 Sloughed cells in tubular lumens in the cortex of a kidney with advanced necrosis, forming casts. The globules of black material are fat. Oil red O. × 540.

4 Interstitial hemorrhage, demonstrated by the presence of numerous red blood cells with widening of the interstitial space between glomerulus and tubules. H & E. × 540.

5 Interstitial hemorrhage, the red blood cells here shown widening the capsule. The black granular material within the tubule near center is calcium. H & E. × 540.

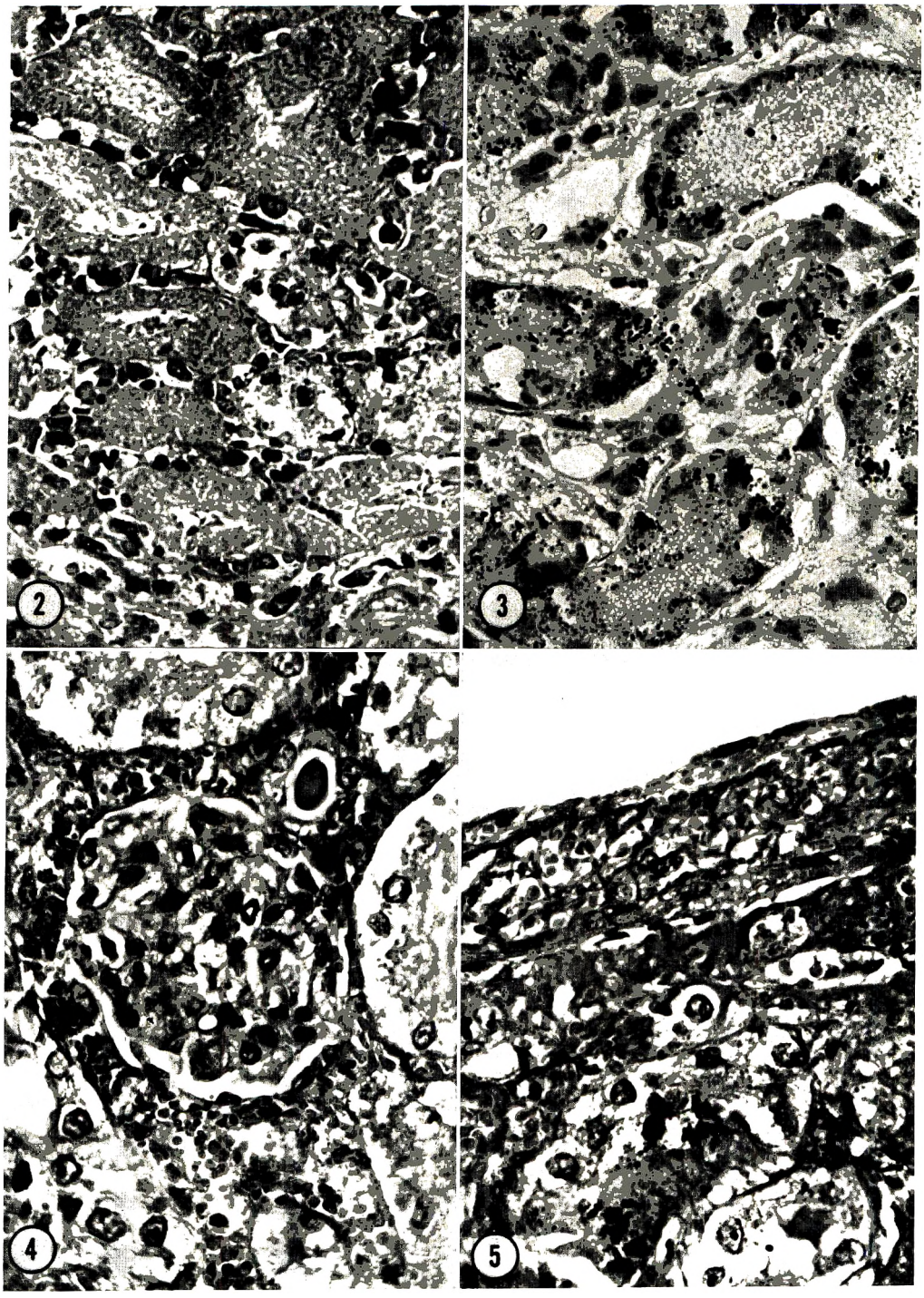
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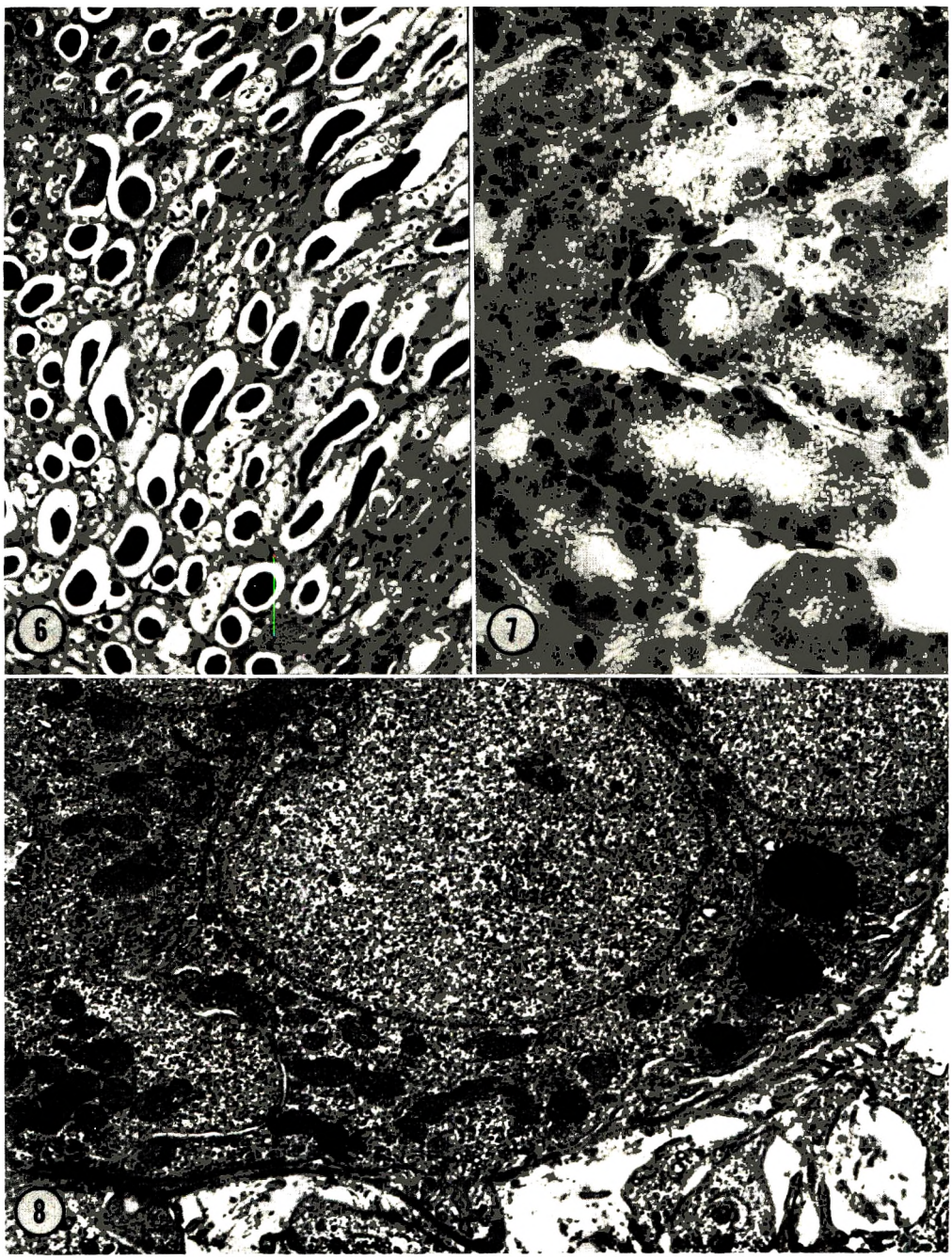
PLATE 2

EXPLANATION OF FIGURES

6 Renal papilla of a rat with advanced necrosis of kidneys. Each collecting tubule contains a deeply staining cast. PAS. × 540.

7 Renal cortex, showing convoluted tubules of a rat that did not develop necrosis but has numerous fat droplets (seen as black) in the epithelial cells. This change was never widespread unless accompanying frank necrosis. Oil red O. × 540.

8 Electron micrograph of a portion of a proximal convoluted tubule near an area of necrosis. No changes are apparent except for the large black fat droplets in the basal portion of a cell. × 12,000.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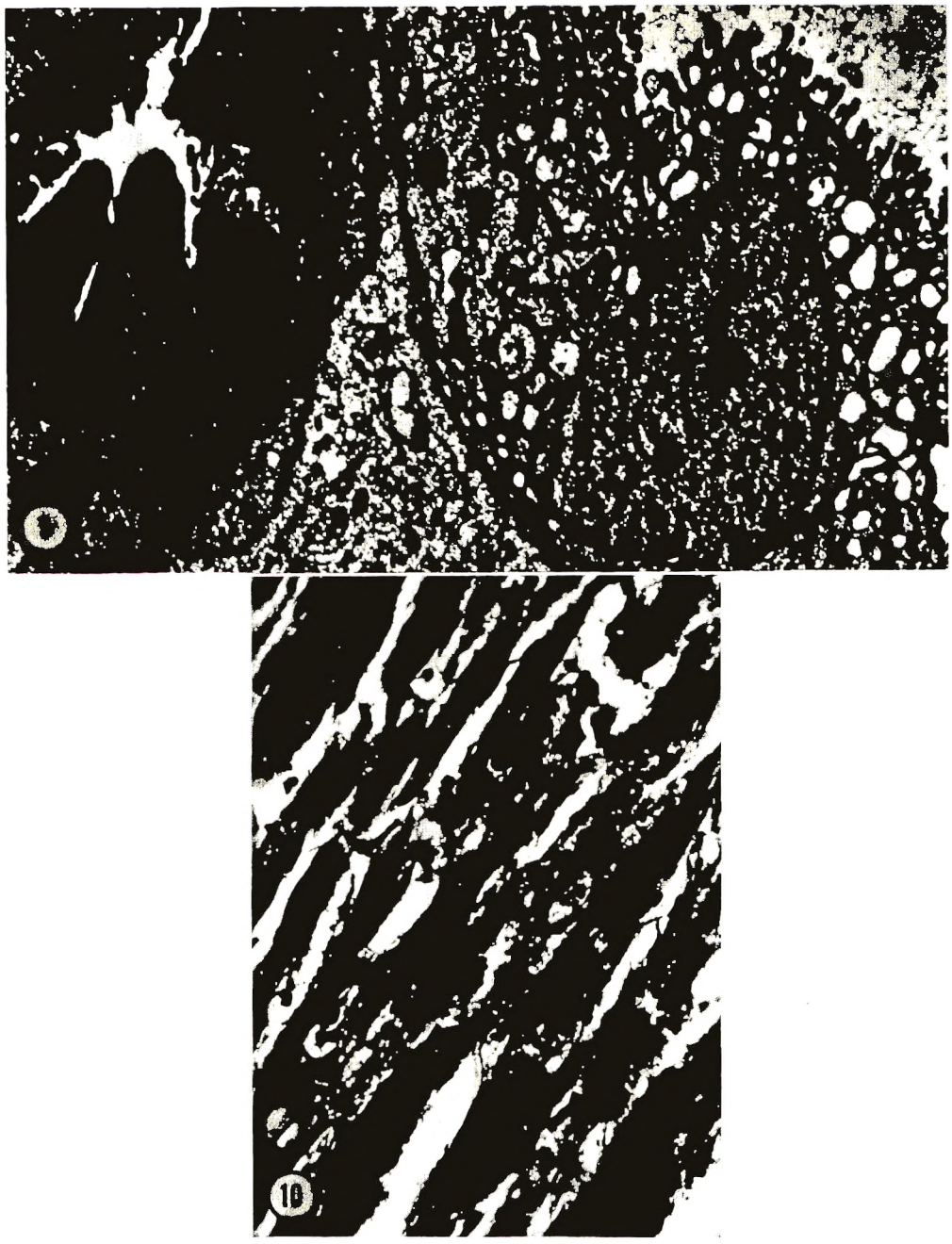

9 Electron micrograph of tubules from another rat showing dilated ergastoplasmic sacs in the apical portion of the cell, near the tubular lumen. An arteriole lies to the left in the interstitial tissue. × 12,000.

10 A focus of myocardial necrosis in a rat that also had advanced renal necrosis. The muscle fibers are partially destroyed (center) and a moderate cellular infiltration is present. H & E. × 540.

Amino Acid Imbalance and Cholesterol Levels in Chicks[1]

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The addition of protein to the dietary regimen as a factor in reducing cholesteremia has received considerable attention (Kokatnur et al., '58a, b; Moyer et al., '56; Jones and Huffman, '56). The higher protein intake may have reduced serum cholesterol levels by correcting a deficiency or an imbalance in amino acids. For example, a deficiency of methionine in the diet of *Cebus* monkeys resulted in higher serum cholesterol levels and atherosclerosis (Mann et al., '53; Fillios and Mann, '54). Similar results were noted in chicks fed methionine-deficient and low-protein diets (Nishida et al., '58; Olson et al., '58). Johnson et al. ('58) noted that amino acid deficiencies influenced serum cholesterol levels in chicks and Seidel et al. ('60) studied the interrelationship of sulfur amino acids and cholesteremia in rats. The effect of an imbalance in amino acids and the importance of nonessential amino acids in cholesteremia has not been evaluated to date, however. The influence of nonessential amino acids on serum cholesterol level may be significant as they constitute an ever present nitrogen source in intact protein. The present study was therefore undertaken to evaluate the effect of nonessential amino acids and the effect of an imbalance in dietary amino acids on serum cholesterol levels.

EXPERIMENTAL

A large population, usually two to three times the required number of day-old male chicks (New Hampshire × Columbian) were fed a complete soybean protein diet (table 1, M) for a period of 7 days, weighed, and the number needed for the experiment selected for uniformity of body weights. The week-old chicks were then randomly divided into groups of 5 chicks each and fed diets which contained either crystalline amino acids or intact protein. The diets which were prepared with crystalline amino acids were based upon either the proportion of essential and nonessential amino acids found in casein, the composition of amino acids found in the fat-free chick carcass or a predetermined composition of essential and nonessential amino acids tested for promotion of optimal growth. The effect of a specific amino acid was studied by excluding it completely from the basal diet or by partial supplementation, as described in the text. The chicks were fed these crystalline amino acid diets ad libitum for a period of 7 days. The intact protein diets (table 1, N) were prepared with soybean oil meal, supplemented with specific amino acids and fed ad libitum for a period of 21 days. Blood

TABLE 1

Composition of pretest basal diet and of the basal diet with intact protein

	Pretest basal diet (M)	Basal diet with intact protein (N)
	%	%
Glucose[1]	57.11	54.80
Soybean meal (50% protein)		38.46
Assay protein[2]	35.30	—
Minerals	5.34	5.34
Corn oil	1.00	1.00
DL-Methionine	0.75	0.20
Glycine	0.30	—
Choline chloride	0.20	0.20
Vitamins	+ +	+ +
	100.00	100.00

[1] Cerelose, Corn Products Company, New York.
[2] ADM C-1 Assay Protein, Archer-Daniel-Midland Company, Cincinnati.

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[1] Presented in part as paper no. 235 at the annual meeting of the Federation of the American Societies for Experimental Biology at Atlantic City, April 15, 1959.

TABLE 2

Composition of amino acid mixture and basal diet[1]

Amino acid	Mixture			
	P[2]	Q[3]	R	S
	% of diet	*% of diet*	*% of diet*	*% of diet*
L-Arginine·HCl	1.52	1.40	2.83	2.30
L-Histidine·HCl	1.12	0.51	1.13	0.92
L-Lysine·HCl	2.95	1.54	2.69	2.19
L-Tyrosine	1.89	0.80	1.51	1.23
L-Phenylalanine	1.74	1.18	1.28	1.04
L-Tryptophan	0.45	0.18	0.36	0.29
L-Cystine	0.12	0.40	0.87	0.70
DL-Methionine	0.99	0.20	0.39	0.32
L-Threonine	1.35	1.16[4]	1.25	1.02
L-Leucine	3.03	1.66	3.64	2.96
L-Isoleucine	1.98	1.68[4]	1.58	1.29
L-Valine	2.22	1.92[4]	1.80	1.46
Glycine	0.63	0.50	1.08	0.88
L-Glutamic acid	15.00	15.00[5]	15.77	12.85
DL-Alanine			1.86	
L-Aspartic acid			1.95	
L-Proline			3.69	
DL-Serine			3.78	
Total	34.99[6]	28.13[6]	47.46	29.45[6]

Ingredients	Basal diets			
	P	Q	R	S
Corn starch	44.47	29.43	31.97	49.98
Minerals	5.34	4.37	5.37	5.37
Cellulose	3.00	3.00	3.00	3.00
Antacid[7]	1.00	1.00	1.00	1.00
$NaHCO_3$	1.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20
Corn oil	10.00	30.00	10.00	10.00
Amino acid mix	34.99	30.00	47.46	29.45
Vitamins	+ +	+ +	+ +	+ +
	100.00	100.00	100.00	100.00

[1] In the formulation of basal diet, the changes in amino acid mixture were made at the expense of starch in the diet.

[2] Equivalent of 30% casein.

[3] Diet devised by G. J. Klain, H. M. Scott, and B. C. Johnson; Poultry Sci., *39:* 39, 1960.

[4] Twice the level of corresponding L-form.

[5] The concentration of L-glutamic acid which was the only source of nonspecific nitrogen was varied to maintain the total amino acids at a constant level of 30%.

[6] Supplements of some or all nonessential amino acids given in these experiments: DL-alanine, 1.86%; L-aspartic acid, 1.95%; L-proline, 3.69%; and DL-serine, 3.78%.

[7] Antacid absorbent (an aluminum hydroxide-magnesium trisilicate preparation) used in free amino acid diets; supplied by Warner-Chilcott Laboratories, Morris Plains, New Jersey.

was drawn by heart puncture from two to 5 representative birds, the blood centrifuged, and the serum stored at − 10°C. Analysis for total serum cholesterol was carried out on individual samples according to the method of Schoenheimer and Sperry ('34).

RESULTS

Amino acids, when added as supplements to diets prepared from crystalline amino acids or intact protein, were observed to have a significant influence on serum cholesterol level in chicks. For example, casein is known to be limiting in arginine for the growing chick. To study the effect of arginine deficiency on serum cholesterol levels, a mixture of crystalline amino acids simulating the proportion of essential amino acids present in a diet containing 30% of casein was prepared and used in the basal diet shown in table

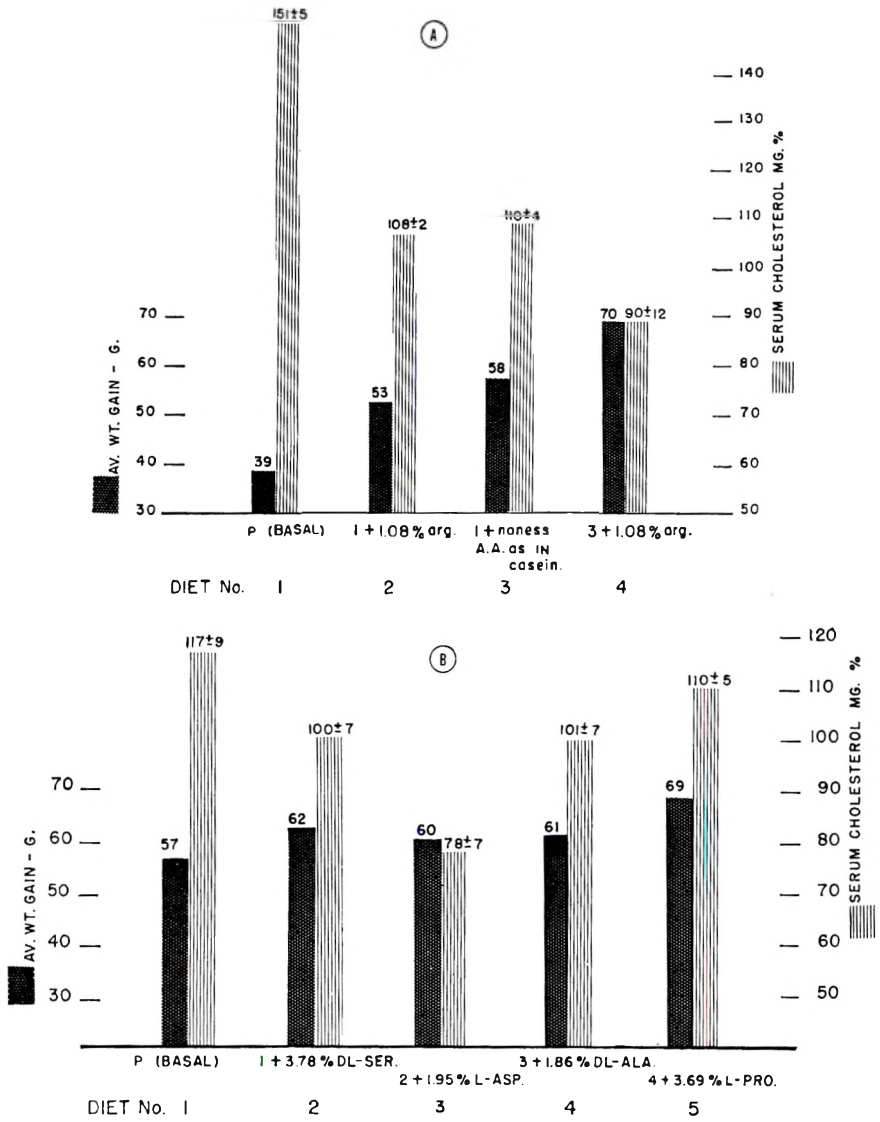

Fig. 1 The effect of (A) arginine and nonessential amino acids simulating 30% of casein and (B) successive addition of serine, aspartic acid, alanine and proline (simulating 30% of casein) on serum cholesterol levels; ± represents standard error of the mean.

2, mixture P.[2] Arginine was excluded in the preparation of the basal mixture. The addition of 1.08% of arginine to the basal mixture improved growth and depressed the serum cholesterol level markedly (fig. 1A). When the basal mixture was supplemented with nonessential amino acids (NEAA) simulating casein, growth was slightly better, and the serum cholesterol level had nearly the same value as in chicks fed 1.08% of arginine. But the addition of arginine and nonessential amino acids to the basal diet significantly improved growth and decreased the serum cholesterol level from 151 to 90 mg per 100 ml.

Because the nonessential amino acids present in intact casein seemed to exert a significant influence on the serum cholesterol level, a separate trial was conducted

[2] Klain, G. J., 1959, Amino Acid Studies with Chicks. Ph.D. Thesis, University of Illinois.

to determine which of the NEAA may have a specific effect. A diet which contained 1.08% of arginine hydrochloride was supplemented with serine or combinations of serine with aspartic acid, alanine, and proline (fig. 1B). A complementary effect of a nonessential amino acid mixture simulating casein was noted in chicks maintained with a mixture which had been supplemented with 1.08% of arginine. For example, when serine was fed in combination with aspartic acid, it effectively lowered the serum cholesterol level to 78 mg per 100 ml (diet 3, fig. 1B). Growth was not significantly affected by the addition of arginine, serine or aspartic acid. The addition of alanine or alanine plus proline did not influence the serum cholesterol levels significantly although growth was slightly improved.

The preparation of another amino acid mixture was based upon the fat-free chick carcass composition given by Price et al. ('53) and further developed by Klain et al. ('60). The proportion of amino acids and the composition of the basal diet is shown in table 2, mixture Q. L-Glutamic acid was added as a source of nonspecific nitrogen to maintain the total amino acids at a constant level of 30% in the diet. Because the chick has a high requirement for leucine, graded levels of this amino acid were added to the basal diet Q devoid of leucine. A deficiency of leucine caused by only partial supplementation of this amino acid resulted in poor growth and a high serum cholesterol value (fig. 2A). When a progressive increment of leucine up to 2.06% was added, which was actually in excess of the required level, a gradual decrease in serum cholesterol was observed. A sudden depression in growth and an elevation in cholesterol value was noted when the leucine level in the diet exceeded the optimal requirement.

The glycine requirement of the chick is still controversial. To study its effect on serum cholesterol levels, increasing levels of glycine were added to the basal diet prepared with mixture Q (table 2) devoid of glycine. Although a slight elevation in cholesterol values was observed (fig. 2B), increasing supplements of glycine did not have any marked influence on serum cholesterol levels or growth.

The nonessential amino acids of casein which were observed to influence serum cholesterol levels were added singly to the basal diet (containing mixture Q, table 2) in the proportion which would be present in a diet containing 30% of casein. Serine, aspartic acid, alanine or proline added individually to the basal diet (fig. 2C) failed to show a significant influence on cholesterol levels. But a marked depression in growth and an elevation in serum cholesterol level was noted in chicks which received 1.86% alanine.

To study the effect of an optimal level of arginine on serum cholesterol level, the proportion of essential amino acids in the mixture (table 2, mixture R) was increased to 2.17 times that in mixture Q (table 2), the specific requirement values reported by Klain et al. ('60). An adequate level of glutamic acid and nonessential amino acids of simulated casein were included in the composition for maximal weight gain. This amino acid, mixture R, was observed to be very efficient and gave growth responses similar to the intact protein.[3] A basal diet prepared with mixture R devoid of arginine was supplemented with increasing amounts of arginine hydrochloride. It was noted that growth performance improved with each increment of arginine until an optional level of arginine was reached (fig. 3A). No significant effect on serum cholesterol levels was observed, however. There was a marked improvement in growth, and the serum cholesterol level was lowered when a deficiency of arginine was partially corrected by the addition of 1.74% of arginine hydrochloride.

To mixture R (table 2) free of lysine or proline graded levels of lysine (free base) or proline were added to determine their effect on cholesterol values. The optimal requirement of lysine for growth may depend upon the efficacy of the amino acid mixture, and its requirement may increase with efficient utilization of other amino acids.[4] It was observed that there was a very marked lowering of serum cholesterol level with maximal growth response when 1.4% of lysine was added to the basal diet (fig. 3B). At a level of 1.8% of lysine, a further decrease in serum cholesterol level

[3] See footnote 2.
[4] See footnote 2.

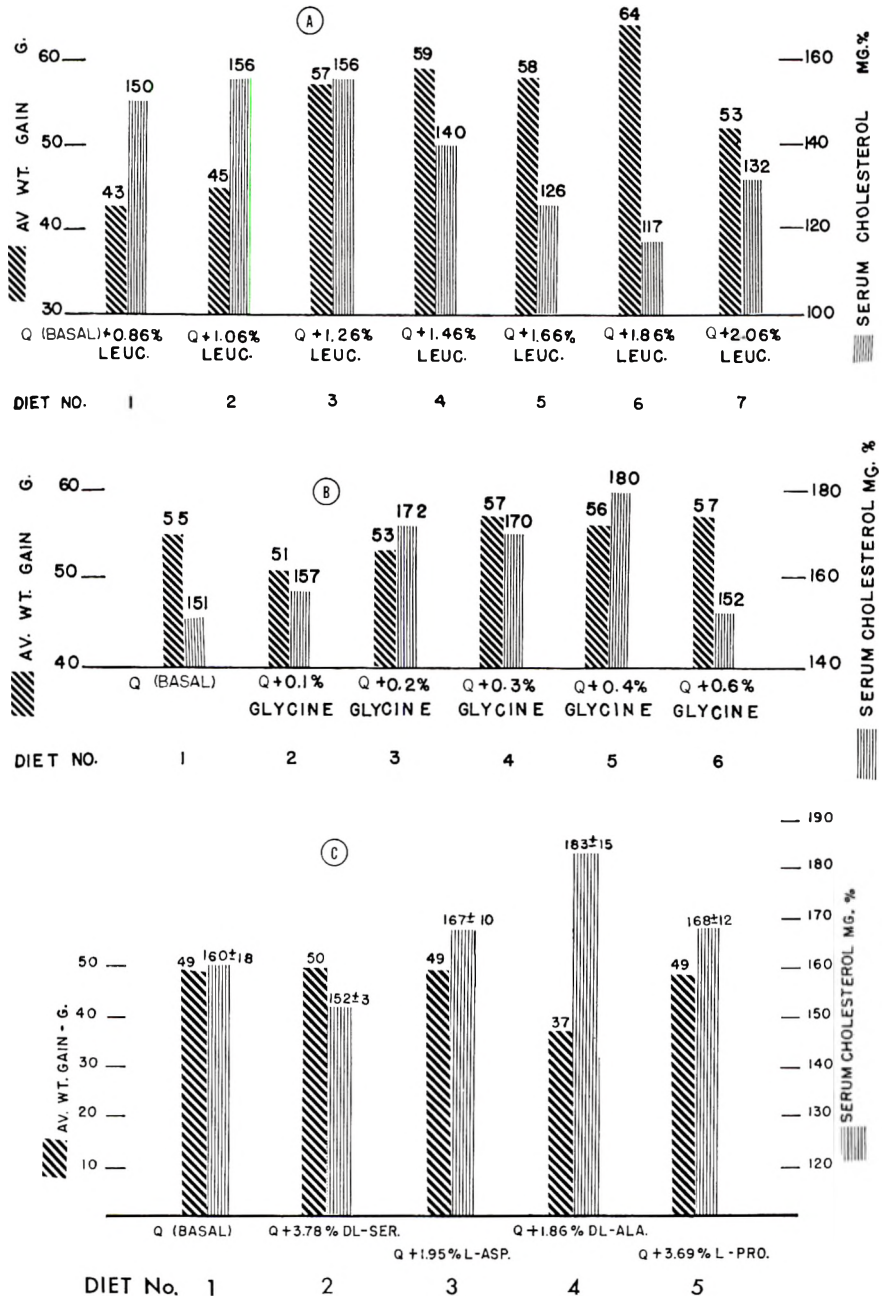

Fig. 2 A, The effect of leucine on serum cholesterol level. Linear regression analysis of cholesterol values (Y-axis) vs. percentage of leucine (X-axis) gave a regression coefficient of -24.3; $Y = 174 - 24.3X$.

B, The effect of glycine on serum cholesterol level. An average of two cholesterol values is recorded. The maximal spread of cholesterol values was 24 mg per 100 ml (diet 2). In most other values the differences were about 10 mg per 100 ml.

C, The effect of nonessential amino acids simulating 30% of casein on serum cholesterol level; ± represents standard error of the mean.

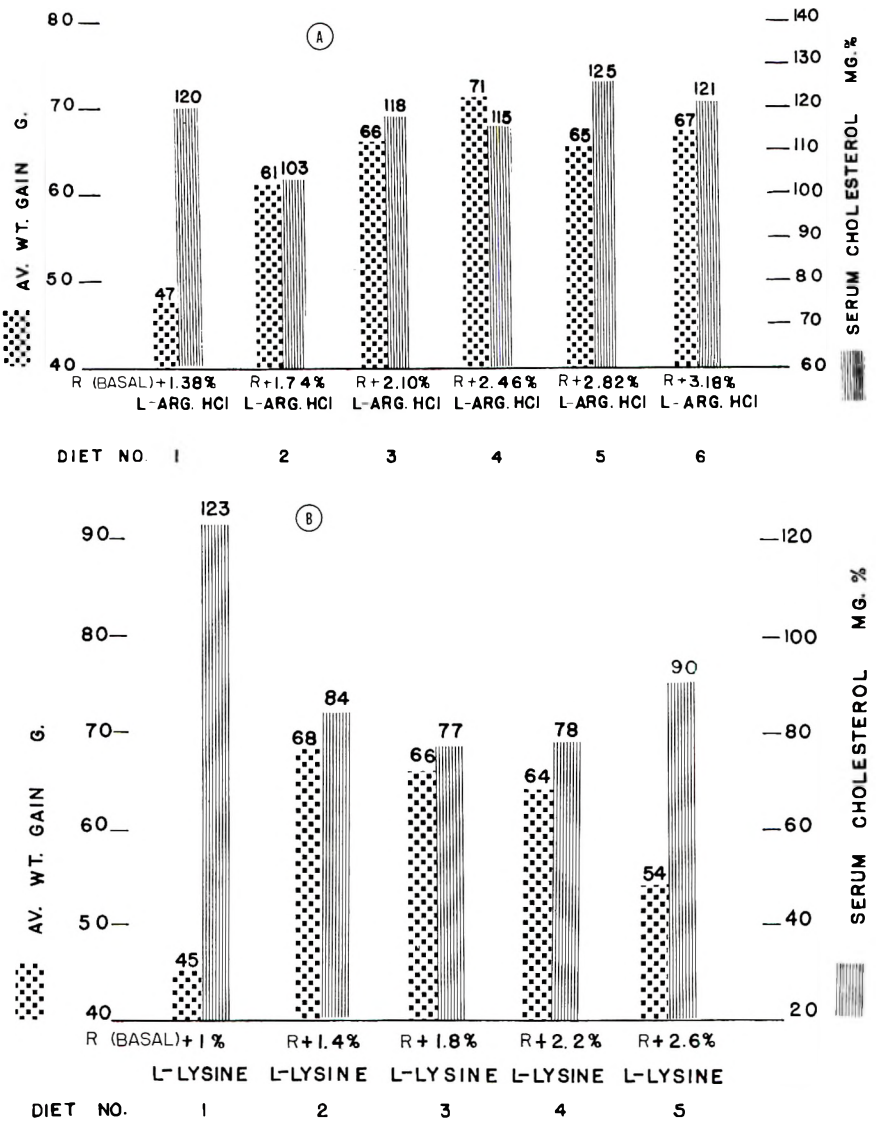

Fig. 3A and B Effect of (A) arginine, (B) lysine on serum cholesterol levels. Average of two cholesterol values is recorded. The maximal spread in values was 14 mg per 100 ml (diet 1A; diet 2B). The difference in most other values was in the range of 5 mg per 100 ml.

and a slight depression in growth occurred. At levels of lysine higher than 1.8% poorer growth response and gradual elevation of cholesterol values were noted. The nonessential amino acid proline did not seem to have any significant influence on cholesterol values (fig. 3C), although there was an improved growth response at a 1% level of proline.

An increase in the level of dietary nitrogen as provided by an elevation in the percentage of the amino acid mixture (table 2, mixture R) in the diet brought about a gradual lowering of serum cholesterol levels (fig. 3D). At a dietary level of 3.2% of nitrogen (protein equivalent 20%) and at 7.2% of nitrogen (protein equivalent 45%), the cholesterol value was 162 and

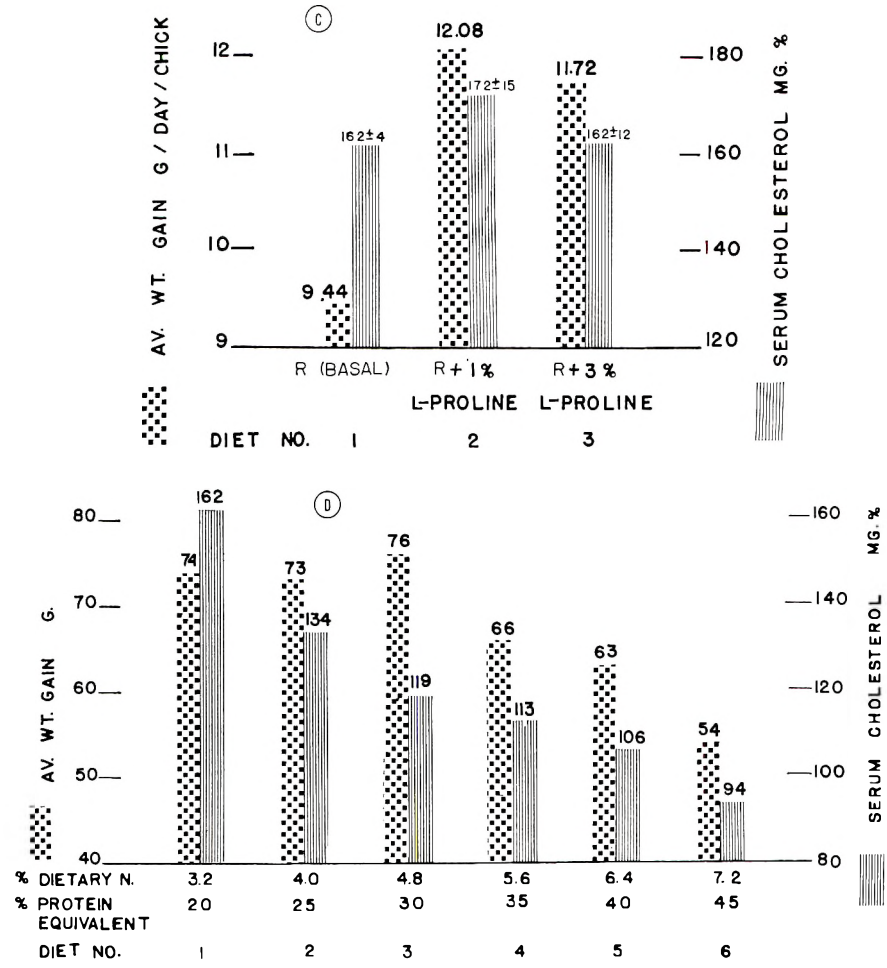

Fig. 3C and D C, Effect of proline on serum cholesterol levels; ± represents standard error of the mean. D, Effect of level of dietary nitrogen on serum cholesterol values. Linear regression analysis of cholesterol values (Y-axis) vs. percentage of protein (X-axis) gave a regression coefficient of -2.5; $Y = 201 - 2.5X$.

94 mg per 100 ml, respectively. Maximal growth response and the most efficient utilization of nitrogen in the chick were noted to occur with a diet containing 4.8% of dietary nitrogen (equivalent to 30% protein).

The influence of nonessential amino acids on the amino acid mixture, shown in table 2, mixture S,[5] indicated that there was a marked lowering of the serum cholesterol level when alanine, aspartic acid and serine were added to the basal diet. When only proline or all 4 amino acids were added to the basal diet, the serum cholesterol value decreased (fig. 4) but was higher than with alanine, aspartic acid and serine. A depression in weight gain was observed in chicks fed diets which contained serine, aspartic acid, alanine and proline, whereas the chicks which received only proline as a supplement showed improved growth response but higher serum cholesterol levels.

An intact protein diet prepared from soybean protein when supplemented with me-

[5] Greene, D. E., 1959, Factors Influencing the Growth of Chicks Fed Crystalline Amino Acid Diets with Special Reference to a Growth Stimulating Factor in Intact Proteins. Ph.D. Thesis, University of Illinois.

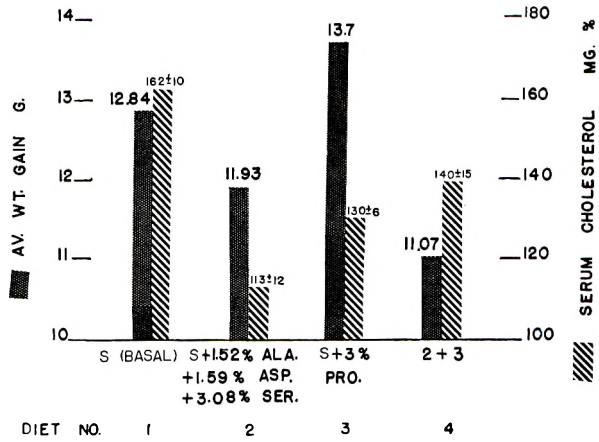

Fig. 4 The effect of alanine, aspartic acid, serine and proline on serum cholesterol levels; ± represents standard error of the mean.

thionine and fed at an adequate level is known to satisfy all the amino acid requirement of chicks (table 1, basal diet N). However, toxicity caused by supplementation with excessive amounts of certain essential amino acids may have in addition to growth depression an influence on cholesterol levels. Three amino acids, lysine, phenylalanine and histidine, were fed at toxic levels to study their effect on serum cholesterol. Since the chicks were selected for uniform weights from a large population, only the average of their final weights was recorded. A significant rise in serum cholesterol value and a marked growth depression was observed when the lysine level was increased from 2 to 4% (fig. 5A). The toxicity caused by supplementation with 3% of phenylalanine did not seem to influence the serum cholesterol level although the same amount of histidine hydrochloride seemed to elevate serum cholesterol level. Nevertheless, the addition of these amino acid supplements did indicate toxicity with respect to growth response.

The effect of certain amino acids in correcting or overcoming the toxicity caused by an excessive amount of lysine is shown in figure 5B in which diet N′ contained 4% of lysine in addition to the basal N (table 1). Glycine, arginine and methionine counteracted the hypercholesterolemic effect of lysine. However, supplements of arginine and methionine did not seem to improve growth. The combined additions of glycine and arginine or glycine and methionine markedly lowered serum cholesterol levels, and a notable increment in weight gain was observed. Antagonism between arginine and methionine was quite apparent. Unusually high serum cholesterol values were observed with this diet, but weight gains were not markedly different. A combined supplement of glycine, arginine and methionine resulted in the lowest serum cholesterol value and best growth.

DISCUSSION

In the young growing chick a proper amino acid balance seems important to a "normal" serum cholesterol level. Crystalline amino acid mixtures which were deficient or made deficient by the elimination of a specific essential amino acid invariably elevated serum cholesterol levels and depressed growth in chicks. Upon correction of the deficiency, improved growth and lower serum cholesterol levels were observed. Since casein is deficient in arginine for optimal chick growth (Arnold et al., '36; Fisher et al., '54), addition of this amino acid to a mixture of essential amino acids simulating casein gave a very favorable response in weight gain and lowered serum cholesterol values. These results are in agreement with those reported by Johnson et al. ('58) when feeding an intact casein diet. The addition of arginine to a mixture of crystalline amino acids did not produce a marked change in serum cholesterol level, however. Similar

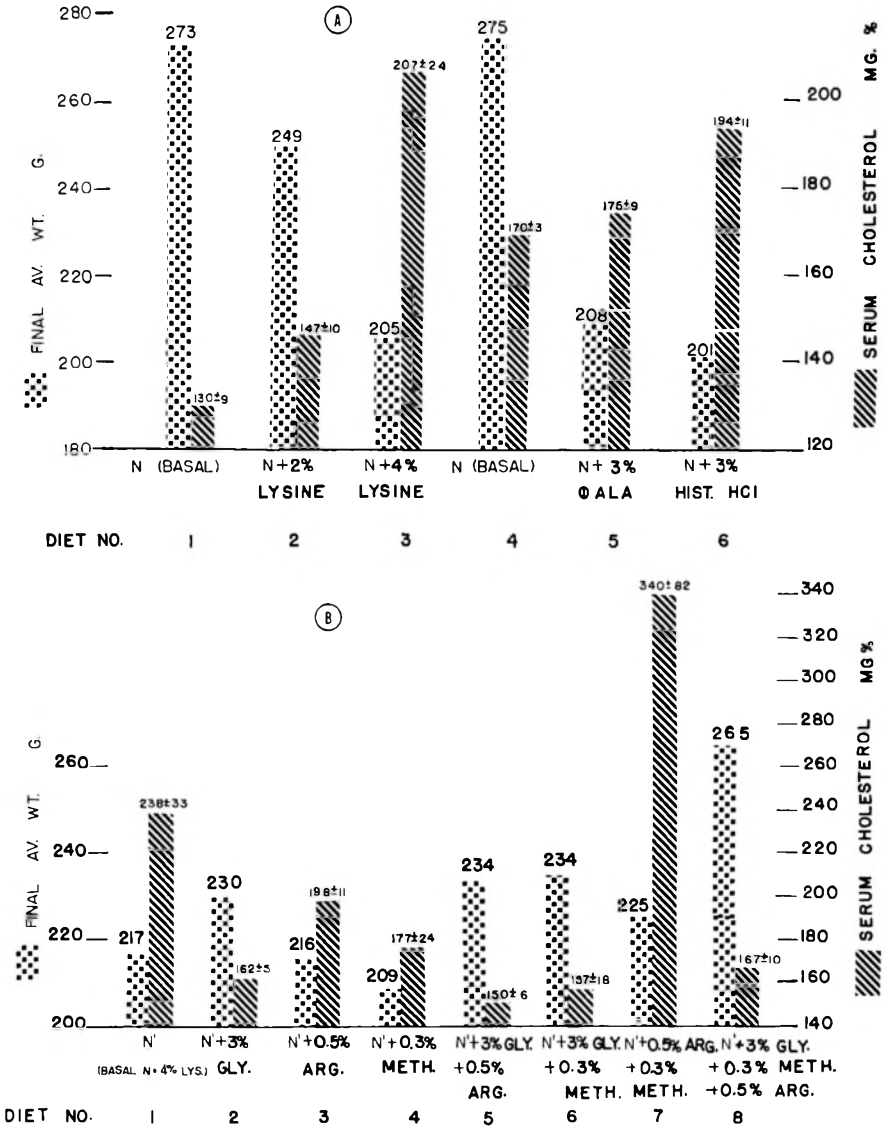

Fig. 5 A, Effect of toxic levels of lysine, phenylalanine and histidine on serum cholesterol values. B, Effect of amino acids in overcoming the toxic effects of lysine; ± represents standard error of the mean.

results were obtained with graded levels of glycine. Therefore, the requirement for a specific amino acid for normal growth and a "normal" serum cholesterol level may depend on the right proportion and proper balance of all the essential amino acids in protein.

The specific amino acid involved in either the depression or elevation of serum cholesterol levels did not necessarily have to be an amino acid previously classified as indispensable. For example, the addition of a mixture of nonessential amino acids which simulated casein generally showed a marked hypocholesterolemic effect although no advantage in weight gain was noted. Individual amino acids in the group of nonessential amino acids were without effect, but in various combinations some amino acids were observed to have a

complementary influence and depressed serum cholesterol levels. Thus, the addition of serine and aspartic acid or serine, aspartic acid and alanine lowered the serum cholesterol level significantly. Addition of proline alone or in combination with other nonessential amino acids seemed to elevate slightly the serum cholesterol level.

The hypocholesterolemic effect of serine and aspartic acid may be related to the uricotelic nature of the species. Von Knieriem (1877) showed that the feeding of various amino acids to hens resulted in increased uric acid excretion. According to Minkowski (1886) and von Mach (1888), loss of uric acid through excretion in the avian species is replaced by lactic acid and urea, and both may act as precursors of uric acid (Wiener, '02). Increased uric acid excretion in mammals supplied with various protein foods, and especially the effect of specific nonessential amino acids such as glycine and aspartic acid have been previously reported by some investigators (Taylor and Rose, '14; Lewis and Doisy, '18; Lewis et al., '18). A stimulation of the uricotelic process may indirectly either decrease the availability of precursors for cholesterol synthesis by increasing the amount of lactic acid and urea in birds or may make precursors less available due to energy-yielding reactions needed for uric acid synthesis in mammals.

Johnson et al. ('58) noted no inverse relationship between serum cholesterol and growth. Our results also indicate the absence of such a relationship (fig. 3D). If the amino acid composition of a protein is not severely deficient in or unbalanced by a slight excess of an essential amino acid, the hypocholesterolemic effects due to an increased nitrogen intake may insure lower serum cholesterol values. However, as growth may be depressed by an excessive intake of dietary nitrogen, the use of protein as a hypocholesterolemic agent in chicks is limited.

A small excess of some essential amino acid causing imbalance in a protein may reflect its quality by poor growth and high cholesterol levels. An intact protein, if supplemented with a large amount of an essential amino acid in which it is not deficient, may cause toxicity and an elevation in serum cholesterol levels. But this may not be true with all of the essential amino acids although poor growth may be exhibited. Also, some amino acids in small supplements may help to overcome toxicity, causing hypercholesterolemia and growth depression, and some may only lower the serum cholesterol level. Certain amino acids may even act as antagonists and elevate the serum cholesterol level excessively, while improvement in growth may not be marked. Thus fortification of intact protein with amino acids may not be beneficial unless their effect on the serum cholesterol level as well as on growth are taken into consideration. Furthermore nonessential amino acids hitherto believed to have no specific role as determined by growth response seemed to possess a hypocholesterolemic effect with varied dietary regimens.

The biological need for a specific amino acid has to date been evaluated either on the basis of growth or nitrogen retention. Although growth characteristics and nitrogen balance have served as the best available criteria in evaluating the protein needs of an animal, it is evident in the context of the present study that the serum cholesterol levels may also reflect the need for a "balanced" dietary amino acid intake.

SUMMARY

Among the amino acids observed to be essential for optimal growth in chicks, 6 have been tested for their effect on the serum cholesterol levels in diets which were either deficient or more than adequate in one of the amino acids. Under these conditions arginine, lysine and leucine influenced the serum cholesterol level as well as growth. Of the amino acids tested, the toxicity produced by a more-than-adequate amount of phenylalanine caused depression in growth but had no effect on serum cholesterol levels. Lysine and histidine elevated serum cholesterol levels and depressed growth. The toxic effect of lysine could be partly overcome by supplements of a mixture of glycine and arginine or glycine and methionine, but arginine and methionine showed antagonism in their influence on cholesterol level. A mere increase in the dietary nitrogen intake effected by manipulating the amount of a balanced amino acid mixture progressively

lowered the serum cholesterol levels. Although not essential for optimal growth, some nonessential amino acids such as serine and aspartic acid in combination, or serine, aspartic acid and alanine decreased serum cholesterol levels in chicks.

ACKNOWLEDGMENT

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Counteraction of the Growth Depression of Raw Soybean Oil Meal by Amino Acid Supplements in Weanling Rats[1]

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Raw soybean oil meal, as the only source of amino acids in an otherwise complete ration, supports a growth rate less than that of a similar ration containing properly heated meal (Osborne and Mendel, '17). Recent reports (Borchers, '58; Hayward, '59) have established that increasing the level of meal to 40% of the ration fed to rats resulted in similar growth rates with the raw and heated soybean oil meal rations. This observation prompted the hypothesis that, at the 25% raw soybean oil meal level, certain of the amino acids were not available in adequate amounts or that the need for certain amino acids was increased for undetermined reasons. The importance of amino acids in the raw soybean oil meal problem has also been implicated in the publication of Fisher and Johnson ('58). These authors reported that a supplement of 14 amino acids was effective in overcoming the growth depressing effect of raw soybean oil meal in chick rations. Booth et al. ('60) have also reported that the addition of 4 specific amino acids to a raw soybean diet corrects the poor growth in rats.

The present report presents the conclusions from our search for a minimum supplement of amino acids necessary to overcome the growth depressing effect of a 25% raw soybean oil meal ration fed to rats.

EXPERIMENTAL

Eleven raw soybean oil meal samples,[2] obtained from commercial soybean processors or prepared by ether extraction of whole soybeans in our laboratory, were used in this study. Because the protein content of the samples ranged from 47.0 to 55.1%, all rations were compounded to contain 12% of soybean protein (N × 6.25) rather than a constant quantity of soybean oil meal. The heated meal was prepared from the raw meal samples by autoclaving in thin layers at 15 pounds steam pressure for 30 minutes. The rations[3] also contained minerals, vitamins, 20% of hydrogenated fat, and starch as used previously (Borchers et al., '57). Further supplements replaced an equal amount of starch from the ration. The rations were fed ad libitum to weanling rats of the Holtzman strain for a 16-day period. Each experiment consisted of two groups of 8 rats in each group, paired as to sex, litter, and initial weight. One group received heated soybean oil meal and the other the raw meal.

RESULTS

Typical growth rates with heated versus raw soybean oil meal rations fed to weanling rats are reviewed in experiments 1–3, table 1. The growth rates with rations containing raw soybean oil meal with no further supplement, with methionine, and with methionine plus antibiotics were 50,

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[2] Samples of soybeans or raw soybean oil meal were graciously supplied by Archer-Daniel-Midland Company, Cargill, Central Soya Company, General Mills, Gooch Milling Company, Spencer Kellogg and Sons, and the Department of Agronomy of the University of Nebraska.

[3] Gifts of certain ration constituents from the Dow Chemical Company, Chas. Pfizer and Company, and Merck Sharp and Dohme are gratefully acknowledged.

TABLE 1

Growth rate of weanling rats fed soybean oil meal rations supplemented with amino acids[1]

Exp.	Soybean sample	Supplement	Soybean oil meal		Raw / Heated	
			Heated	Raw		
			gm gain/day ± SE (gain/food)		%	
1	1	None	3.61 ± 0.15 (0.39)	1.79 ± 0.17 (0.22)	50	8.019[3]
2	1	0.6% DL-Methionine	4.07 ± 0.09 (0.45)	3.04 ± 0.19 (0.37)	75	4.835[3]
4	3	0.6% DL-Methionine 0.1% Procaine penicillin 0.1% Streptomycin sulfate	4.41 ± 0.15 (0.43)	4.17 ± 0.13 (0.46)	95	1.165
3	1	0.6% DL-Methionine 0.6% DL-Threonine 0.5% DL-Valine	3.99 ± 0.24 (0.44)	3.98 ± 0.26 (0.43)	100	0.025
5	1	As "4"	4.14 ± 0.19 (0.46)	3.68 ± 0.12 (0.44)	89	2.011
6	1	As "4" less methionine	3.74 ± 0.15 (0.39)	2.10 ± 0.15 (0.24)	56	7.741[3]
7	1	As "4" less threonine	4.25 ± 0.20 (0.44)	2.82 ± 0.20 (0.38)	66	5.079[3]
8	1	As "4" less valine	4.32 ± 0.17 (0.47)	3.34 ± 0.25 (0.39)	77	3.308[3]
9	9	As "4"	4.13 ± 0.10 (0.43)	3.52 ± 0.17 (0.38)	85	3.049[3]
10	10	As "4"	4.37 ± 0.18 (0.45)	3.11 ± 0.14 (0.32)	71	5.658[3]
11	11	8 Amino acids[2]	4.18 ± 0.17 (0.45)	4.33 ± 0.14 (0.49)	104	0.703
12	9	As "11"	4.33 ± 0.18 (0.43)	4.02 ± 0.16 (0.48)	93	1.132
13	11	As "11" less lysine	4.23 ± 0.17 (0.41)	2.99 ± 0.19 (0.37)	71	4.708[3]
14	11	As "11" less lysine	4.21 ± 0.17 (0.45)	3.69 ± 0.24 (0.40)	88	1.769

[1] Each experiment consisted of two groups of 8 weanling rats each, paired as to litter, sex, and initial weight. The growth rate is expressed as average daily gain for a 16-day period. The rations contained soybean oil meal at a level of 12% of soybean protein (N × 6.25).

[2] Contained (in per cent): L-histidine·HCl, 0.3; DL-isoleucine, 0.4; L-leucine, 0.4; L-lysine·HCl, 0.4; DL-methionine, 0.6; DL-threonine, 0.6; DL-tryptophan, 0.2; and DL-valine, 0.5.

[3] Difference significant at the 1% confidence level.

75, and 95%, respectively, of the control ration containing heated meal plus the same supplement.

When a supplement of methionine, threonine and valine was added, the growth rates with the raw soybean oil meal rations were 89 to 100% of the control heated meal rations for 8 of the samples of soybean oil meal. Each soybean sample was fed in at least two experiments. None of the differences observed were statistically significant. The smallest and greatest difference in results between heated and raw soybean oil meal for the 8 samples are presented in experiments 4 and 5, table 1. Omission of any one of the three amino acids from the supplement resulted in a lower growth rate with the raw soybean oil meal ration. Typical results of each of these are shown as experiments 6–8, table 1.

In three of the soybean oil meal samples, the supplement of methionine, threonine, and valine gave growth rates with the raw soybean oil meal from 71 to 85% of the corresponding control-fed heated meal. Again, each sample was fed in at least two experiments; all differences noted were statistically significant at the 1% confidence level. The smallest and greatest difference observed for these three soybean samples are presented as experiments 9 and 10, table 1. However, a supplement of 8 of the essential amino acids—histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, and valine—resulted in similar growth rates with the raw and heated soybean oil meal rations, using the latter three soybean oil meal samples. Duplicate experiments with each meal gave values ranging from 93 to 104% of the control, experiments 11–12, table 1. Feeding experiments, designed to test each of the 5 additional amino acids to determine whether each was necessary in the supplement, gave inconclusive results which could not be replicated consistently. An example of such data is presented for the omission of lysine, experiments 13–14, table 1. In at least one of the experiments in which one of the latter 5 amino acids (histidine, isoleucine, leucine, lysine, and tryptophan) was omitted, the growth rate with the raw soybean ration was not statistically different from the control. If the data from such experiments were combined, the results would support the conclusion that each of the 8 amino acids was necessary for the latter three soybean meal samples. The variability of the results, however, must be recognized since this variability may be involved in the explanation of the effect of amino acid supplements on growth with raw soybean oil meal rations.

DISCUSSION

These results demonstrate that the primary nutritive factor involved in the raw soybean problem is the supply of dietary amino acids. This conclusion confirms that of Fisher and Johnson ('58) and Booth et al. ('60). The observation that only three amino acids were required in supplementary amounts for 8 of the 11 samples of soybean oil meal suggests that not all of the amino acids are involved or at least not to the same degree. These observations do not yet distinguish between the two general possibilities of why additional amino acids are needed, namely, whether the problem is one of increased requirement or decreased availability. There are several pertinent observations which argue against the possibility of decreased availability, an idea which is supported only by the existence of a trypsin inhibitor (Ham et al., '45) in raw soybeans. First, the gross digestibility of soybean protein from raw meal has been shown to be similar to the heated meal (Hayward et al., '36; Johnson et al., '39; Borchers et al., '47). Second, the feeding of a purified trypsin inhibitor was shown to have an innocuous effect on growth both for chicks and rats (Borchers et al., '48). Third, crude extracts of soybean and of lima bean have been shown to reduce the growth rate when fed with hydrolyzed protein (Westfall et al., '48; Klose et al., '48). And 4th, supplements of antibiotics have been observed to result in similar growth rates when using raw and heated soybean oil meal rations (Borchers et al., '57; Hill et al., '57; Braham et al., '59). This latter effect with dietary antibiotics has generally been regarded as resulting from a sparing effect on various nutrients (Linkswiler et al., '51); that is, antibiotics reduce the apparent requirement for, but do not in-

crease the digestibility of nutrients. The observation that higher levels of raw soybean oil meal supported growth rates similar to those of heated meal (Borchers, '58; Hayward, '59) does not differentiate between increased requirement for or reduced availability of amino acids since either condition would be corrected by an increase in the total amino acid intake.

If the problem is one of an increased requirement for certain critical amino acids, such an increase could result from several possible situations. First, pancreatic stimulation (Lyman and Lepkovsky, '57) by the raw soybean trypsin inhibitor has been suggested by Booth et al. ('60) to result in excessive loss of certain critical amino acids via fecal excretion of pancreatic enzymes. This suggestion is contradicted by the innocuous effect on growth of a purified trypsin inhibitor and by the effect of antibiotics cited in the previous paragraph. Second, raw soybeans may contain a heat-labile toxic factor which interferes with the metabolism of certain of the amino acids or certain amino acids are required for the detoxication of such factors. Third, the reduced growth rate might result from an enhanced deleterious bacterial activity in the gut when raw soybeans, or fractions thereof, are fed. The possibility for such a situation is supported by the observations with antibiotic supplements to raw soybean rations (previous citations). Such deleterious bacterial activity in the gut could involve activities ranging from a simple destruction of amino acids to an increased need for amino acids in detoxication of bacterial degradation products. The apparent variable response to omission of one amino acid from the supplement of 8 amino acids with the three soybean samples would seem to suggest that such variability might result from the variable intensity of bacterial action in successive experiments.

SUMMARY

Raw soybean oil meal, when fed to weanling rats at a 12% protein level as the sole source of amino acids, supported a growth rate less than that observed with the use of heated soybean oil meal. Supplementing the ration with methionine, threonine, and valine resulted in an increased growth rate with the raw soybean oil meal ration. With 8 of the 11 soybean samples, this supplement supported growth rates which were similar for raw and heated rations. With three of the 11 samples, a supplement of 8 essential amino acids supported similar growth rates. The results are interpreted as an indication that the feeding of raw soybean oil meal causes an increased apparent dietary requirement for amino acids. Possible mechanisms for such an increased requirement are pointed out. The case against decreased availability of amino acids from raw soybeans is reviewed.

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Studies on the Kidney in Vitamin E Deficiency[1]

II. RENAL TOCOPHEROL CONTENT IN RELATION TO VITAMIN E DEFICIENCY CHANGES IN THE KIDNEY

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In rats fed from weaning a diet deficient in vitamin E and rich in long-chain unsaturated fatty acids, alterations occur in the kidney resulting in an increased rate of postmortem autolysis (Emmel, '57; Moore et al., '57, '58). Under the experimental conditions previously reported (Emmel, '57) an interval of about 6 weeks elapses before the onset of this change. It has been presumed that this interval represents the time required for reduction of the animal's tocopherol stores to a critically low value, although there are no data in the literature to indicate the rate at which tissue tocopherols decline in rats deprived of vitamin E. The present study was undertaken to determine the relationship between renal tocopherol content and the onset and progress of the renal change associated with these dietary influences. Observations here reported demonstrate that the renal tocopherol level falls rapidly on the vitamin E-deficient regimen and reaches a minimal value approximately coincident with the onset of the above renal abnormality.

MATERIAL AND METHODS

Rats of both sexes, reared by mothers fed a commercial stock ration,[3] were weaned at 21 days and supplied with a vitamin E-deficient diet[4] in which the caloric distribution was carbohydrate, 46%; protein, 24%; and fat, 30%; the latter consisted of the free fatty acids of linseed oil (Emmel, '57). The tocopherol content of the complete diet, by analysis, was 3.4 μg per gm of diet. Vitamin A (400 U) and vitamin D (40 U) were given orally twice weekly to each animal. Animals were decapitated after receiving the experimental diet for intervals up to 25 weeks. Kidneys were promptly excised, decapsulated and sectioned longitudinally. Hilar fat and the renal papilla were carefully removed and the kidneys frozen and stored for about 24 hours in the freezing compartment of a refrigerator. Tissue specimens were extracted at room temperature with Skellysolve-F; tocopherols were isolated by molecular distillation (Quaife and Harris, '48; Quaife and Dju, '49) and total tocopherols in the distillate were estimated colorimetrically by the method of Emmerie and Engel ('38).

The Skellysolve-F used for the extractions was purified by shaking in a separatory funnel with silver nitrate, dehydrating over calcium chloride, and finally distilling in a glass still. Ethanol was purified by distilling from sodium hydroxide and ferric nitrate.

Extracts were prepared by triturating the specimens with anhydrous Na_2SO_4 and

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[2] Some of the data in this report form part of a thesis (P.L.L.) submitted in partial fulfillment of the requirements for the degree, M.D. with Honor.

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

[4] Composition of the diet: (in grams) crude casein (Borden Co.), 238; dextrose (Cerelose, Corn Products Co.), 489; dried brewer's yeast (type 2019; Standard Brands, Inc.), 89; salt mixture (H.M.W.; Nutritional Biochemicals Corp.), 30; linseed fatty acids, 155. The linseed FA was obtained from the Woburn Chemical Corp.; Harrison, N. J. (product no. 441). It was stabilized against oxidation by adding 0.1% each of citric acid and propyl gallate. Its peroxide value of 2 to 6 mEq per kg remained unchanged after exposure of the diet to room temperature for 24 hours.

transferring to stoppered 50-ml graduates to which were then added 25 ml of Skellysolve-F, 8 ml of ethanol and 4 ml of distilled water. The mixtures were shaken vigorously for 10 minutes, then allowed to stand in the dark for 0.5 hour to permit separation of solvents. Aliquots (20-ml) of the clear supernatant extracts were evaporated to dryness in the previously weighed aluminum cups of the molecular still. Evaporation was carried out on a steam table under a continuous stream of nitrogen, and care was taken to assure an even distribution of the lipid residue during evaporation of the solvent. After cooling for 5 minutes the cups were weighed to determine the amount of lipid extracted. The sample cups were then placed in the molecular still and distillation allowed to proceed for one hour at a temperature of 215 to 220°C and a pressure of 1 to 5 μ Hg. The distillates were then rinsed from the condensers with 5 ml of warm purified ethanol. A 4-ml aliquot of the alcoholic solution of each distillate was assayed colorimetrically by the Emmerie-Engel procedure, using 2 ml each of alcoholic solutions of 2-2′ bipyridine (200 mg per 100 ml) and $FeCl_3$ (80 mg per 100 ml). Exposure to light after adding the bipyridine was kept to a minimum, and 15 seconds after adding the $FeCl_3$ optical densities were determined on an Evelyn colorimeter, using a 515 mμ filter.

A reagent blank was determined for each group of 4 analyses. Tocopherol concentration in the alcoholic solution of molecular distillate was determined from a calibration curve plotted from analyses of standard solutions of pure α-tocopherol. The analytical values were finally expressed in terms of tocopherol concentration in the extracted lipid and in the initial fresh tissue.

In the work here reported hydrogenation was not used to eliminate possible interference by vitamin A in the Emmerie-Engel reaction. Preliminary tests showed that hydrogenation did not abolish the reaction obtained in distillates from the long-term vitamin E-deficient animals. It was further observed that the addition of vitamin A did not produce detectable interference until about 10 times the amount which might reasonably be expected in rat kidney (Eden and Moore, '51) had been added. Thus it was felt that vitamin A could be disregarded in carrying out these analyses. Scheduling of the experiments was such that each animal received its last oral supplement of vitamins A and D 24 hours before tissues were removed for analysis.

Lipid peroxides in adipose tissue of another series of animals subjected to the above vitamin E-deficient dietary regimen were determined by a slight modification of the method of Hartmann and Glavind ('49), using leucodichlorophenolindophenol as reagent. Samples of adipose tissue weighing about 0.5 gm were promptly removed and triturated with about 5 gm of anhydrous Na_2SO_4 and a few milligrams of a mixture containing equal amounts of citric acid and propyl gallate. The latter did not alter the blank values and served to prevent a rise in peroxides during analysis. Toluene was used both as lipid extractant and as solvent in the colorimetric determination. A suitable aliquot of extract was diluted to 10 ml with toluene, and to it were added 0.5 ml of glacial acetic acid and 0.2 ml of the above reagent (0.2% in 100% alcohol). The mixture was heated to 100°C for 10 minutes, then cooled to room temperature. The optical density of the red color developed was determined with an Evelyn photoelectric colorimeter using a 515 mμ filter. A second aliquot of extract was evaporated to dryness for weighing. Lipid peroxide values were calculated as milliequivalents of peroxide per kilogram of lipid (mEq/kg). Since both the peroxide value and appearance of the adipose tissue varied widely at different sites in the body, the sample for analysis was usually made up by combining representative samples of fat from subcutaneous, retroperitoneal and paragonadal sites. In some instances individual samples were analyzed separately and averaged for tabulation.

RESULTS

Tocopherol analyses. Kidneys from 55 animals fed the vitamin E-deficient diet were analyzed for their total tocopherol content. Data from 39 of these were recorded individually (table 1); data for kidneys from the remaining 16 younger

TABLE 1

Renal tocopherol during vitamin E depletion in the rat

Time fed diet[1]	Tocopherol concentration									
	Individual animals				Av.	Individual animals				Av.
	mg/gm lipid extract					*mg/100 gm tissue*				
0[2]					0.51					1.29
2 Days[3]					0.47					0.92
1 Week[2]					0.25					0.62
2 Weeks	0.37	0.18	0.23	0.10	0.22	0.69	0.34	0.48	0.15	0.41
3 Weeks	0.11	0.14	0.14	0.09	0.12	0.22	0.30	0.32	0.19	0.26
4 Weeks	0.08	0.09	0.05	0.09	0.09	0.18	0.22	0.12	0.21	0.18
5 Weeks	0.14	0.14	0.12	0.11	0.12	0.17	0.28	0.24	0.20	0.23
6 Weeks	0.04	0.12	0.13	0.04	0.08	0.09	0.30	0.29	0.12	0.20
7 Weeks	0.06	0.09	0.05	0.06	0.07	0.16	0.21	0.12	0.17	0.17
9 Weeks	0.07	0.13	0.06	—	0.09	0.18	0.30	0.13	—	0.20
12.5 Weeks	0.10	0.12	0.17	0.08	0.12	0.28	0.38	0.40	0.22	0.32
15 Weeks	0.17	0.03	0.03	0.07	0.08	0.45	0.16	0.06	0.18	0.21
25 Weeks	0.04	< 0.02	0.12	0.06	0.06	0.08	< 0.04	0.27	0.12	0.12

[1] Animals weaned and fed vitamin E-deficient diet at 21 days of age.
[2] Pooled specimens from 5 rats.
[3] Pooled specimens from 6 rats.

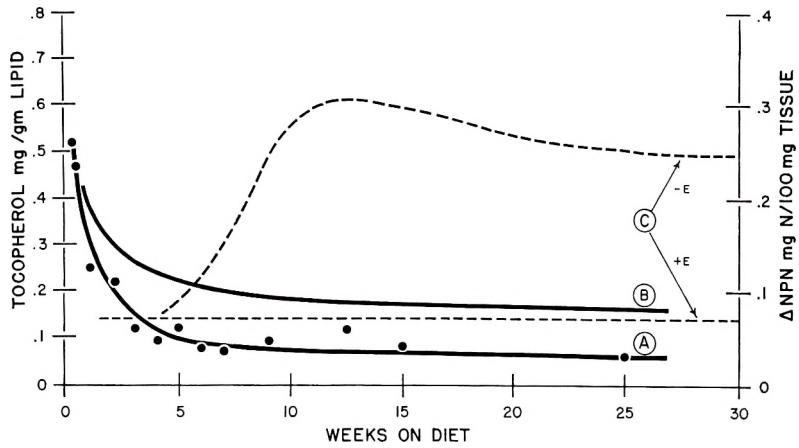

Fig. 1 Tocopherol and autolysis in rat kidney (linseed FA diet). Animals started to receive the vitamin E-deficient diet at 21 days of age. A, renal tocopherol expressed as milligrams per gram of extracted lipid; B, renal tocopherol concentration as hypothetically influenced by increase in organ weight during growth of the animal; C, postmortem autolysis in the kidney expressed as NPN liberated by 100 mg of tissue during incubation at 37° for 6 hours (Emmel, '57).

animals were grouped to give combined samples of sufficient size for analysis. The average values expressed as milligrams of tocopherol per gram of extracted lipid are plotted in figure 1A. The renal tocopherol concentration declines rapidly on the vitamin E-deficient regimen, and its minimal value of about 0.08 mg per gm of lipid is attained after about 6 weeks of feeding the diet. This corresponds very closely with the time at which the renal autolytic rate begins to rise significantly above normal (fig. 1C). Analytical data from 8 stock animals fed a standard commercial diet are shown in table 2. The average value for the kidneys of the entire group is 0.38 mg per gm of lipid, a figure slightly lower than that obtained for weanling animals, but approximately 6 times the value for animals fed the vitamin E-deficient diet 6 weeks or longer.

Average values for tocopherol in terms of wet weight of tissue obtained from both weanling and normal adult animals agree quite well with data reported by others. Quaife et al. ('49) noted 1.18 mg of tocoph-

TABLE 2

Tocopherol in kidney and adipose tissue of rats fed stock diet[1]

No. animals	Age	Kidney		Adipose tissue	
	weeks	*lipid extract mg/gm*	*fresh tissue mg/100 gm*	*lipid extract mg/gm*	*fresh tissue mg/100 gm*
8	9–25	0.38 (0.28–0.53)[2]	1.01 (0.80–2.38)	0.17 (0.10–0.25)	12.7 (8.45–15.5)

[1] Purina Fox Chow, Ralston Purina Company, St. Louis.
[2] Figures in parentheses show range of values.

TABLE 3

Fat peroxides in adipose tissue of rats supplied with vitamin E-deficient diet at three weeks of age

Weeks fed diet	No. animals	Peroxide values	
		Range	Average
		mEq/kg	*mEq/kg*
0	6	(Pooled)	0
2	6	(Pooled)	6.5
3	5	0–82	16
4	5	0–85	31
5	14	0–54	26
6	12	0–350	168
7	17	0–2300	686
9	13	13–2078	327
11	16	33–1150	252
13	6	71–875	205
15–16	11	18–460	82
18–20	6	78–450	234
22–25	7	120–880	491
37	1	—	31
58	1	—	9
68–70	4	—	0
72	1	—	166
77	1	—	0

erol per 100 gm of kidney in a rat receiving 1 mg of α-tocopherol daily. Average values reported for human kidney are 0.80 mg per 100 gm (Quaife and Dju, '49) and 0.68 mg per 100 gm (Dju et al., '58). If one accepts the mean fertility dose for the rat as 0.75 mg of *d*-α-tocopherol (Mason and Harris, '47), then Mason's ('42) bioassay data can be calculated to yield a renal tocopherol content of 1.25 mg per 100 gm of tissue, a figure in remarkable agreement with subsequent chemical analyses.

While the present manuscript was in preparation, additional data became available from the extensive studies by Edwin et al. ('61) on tocopherol distribution in rat tissues under various conditions of dietary intake. There is good agreement between their data and ours on the tocopherol content of kidney and adipose tissue of animals fed an adequate stock diet. Although our vitamin E-deficient diet had a significantly different fat content than theirs, the renal tocopherol levels in our depleted animals were quite similar to those reported by the above authors for depleted male rats, but somewhat lower than those for their female rats. Tocopherol depletion in the adipose tissue of our animals occurred very rapidly: in 16 animals fed the diet for 4 weeks or longer the average tocopherol concentration was 0.003 mg per gm of lipid (cf. table 2). This figure is lower than the value reported by the above authors, and probably reflects the influence of the highly unsaturated fat included in our diet.

Peroxide values. Lipid peroxide values for the adipose tissue from 120 rats are shown in table 3. It is obvious that this value is an exceedingly labile characteristic of the adipose tissue in an animal undergoing tocopherol depletion in the

presence of polyunsaturated fatty acids. Great variations occurred among animals of similar age, and at different sites within a single animal. Serial biopsy studies on individual animals also gave quite variable results. Since the range of values was so great, no attempt was made to treat the data statistically; but averages were calculated chiefly to give some indication of the distribution of values within the range for each group. In general lipid peroxides make their appearance in the adipose tissue in small amounts quite early during tocopherol depletion. The peak values at 7 to 9 weeks occur at a time when the rate of postmortem autolysis in the kidney is rising rapidly, but precede the attainment of a maximal renal autolytic rate by 3 to 4 weeks. The peroxide values decline to low levels in the deeply pigmented adipose tissue of a majority of animals fed the diet for a year or more.

The high peroxide values noted in our animals are undoubtedly related to the high intake of linolenic acid provided by the diet. Aaes-Jørgensen et al. ('51) observed values of 0 to 55.5 mEq per kg for animals fed for 10 weeks a vitamin E-deficient diet containing 20% of cod liver oil. Dam et al. ('52) reported an average value of 213 mEq per kg for animals receiving a similar diet for 14 weeks, a result close to our average value of 205 mEq per kg at 13 weeks. From the rise and decline in lipid peroxides here reported it would appear that peroxides initially build up to a high value prior to extensive polymerization, and that subsequently formed peroxide then combines with the existing polymer and thus does not again attain as high a concentration.

DISCUSSION

The data which have been presented demonstrate that weanling rats fed a vitamin E-deficient diet suffer a rapid decline in renal tocopherol concentration. This in turn may be presumed to reflect a concomitant reduction in tocopherol stores throughout the body. It would appear significant that most of this decline occurred prior to the onset of the increased rate of renal postmortem autolysis. Although peroxidation in the adipose tissue does not necessarily mean that a corresponding change is occurring in protoplasmic lipids, it is noteworthy that lipoperoxides appear in the adipose tissue before the onset of renal change, and build up to a maximum during the development of the renal abnormality. Furthermore, in individual animals increased renal autolysis was noted only in those animals in which lipid peroxides had already made their appearance. These time relationships are consistent with the view that the basis of the increased renal autolytic rate may lie in an altered state of the unsaturated fatty acids incorporated into phospholipids and lipoproteins including the membranes of such cytoplasmic particulates as the mitochondria and lysosomes. It should not be inferred that the accelerated autolytic rate is directly dependent upon a low tocopherol level per se, since therapeutic restoration of the tocopherol level to normal does not concomitantly return the renal autolytic rate to a normal value.[5]

Even after the animals had received an exceedingly low intake of vitamin E for 25 weeks, an appreciable concentration of tocopherol still persisted in the kidney. That this is not an analytical artifact was verified in an analysis of the combined kidneys from two rats which had been fed the vitamine E-deficient diet for 16 months and had a renal tocopherol concentration of 0.037 mg per gm of lipid. By chromatography on paper impregnated with mineral oil and developed in 75% ethanol, this material was identified as α-tocopherol.[6] Edwin et al. ('61) also found α-tocopherol still present in the tissues of rats maintained for 6 months with a vitamin E-deficient diet.

In interpreting results expressed as concentration it must be kept in mind that growth of the animal also may contribute to an observed decline in tissue concentration. Curve B in figure 1 represents the hypothetical effect which growth of the kidney would have had on its tocopherol concentration if its total tocopherol content had remained unchanged. Comparing curves A and B, although the final observed tocopherol concentration is only 12% of the initial value, about 75% of

[5] Emmel, V. M., and P. L. LaCelle, unpublished data.

[6] LaCelle, P. L., unpublished data.

this decline could have resulted merely from increase in size of the kidney. Or, viewed in another way, the kidney at 25 weeks still has a total tocopherol content equal to about 38% of its initial value, the more impressive reduction to 12% of its initial concentration being in part accountable for by growth.

Although there are as yet no data concerning the relative rates of tocopherol loss or movement from one site to another in an animal deprived of this vitamin, it is apparent that a minimal, metabolically inadequate, tissue concentration persists for a long period of time. This may represent the level at which balance is established on the very low intake of tocopherol obtained from the diet, and suggests a tenacity on the part of the depleted animal in absorbing and retaining tocopherol from even this minimal source.

SUMMARY

1. Kidneys from 55 rats supplied with a vitamin E-deficient diet at 21 days of age were analyzed for tocopherol at intervals up to 25 weeks.

2. The tocopherol level fell rapidly during the initial 3 to 4 weeks and reached a minimal value at about 6 weeks. This latter time coincided with that at which the renal autolytic rate began to rise above normal.

3. Minimal levels of tocopherol persisted in the kidneys of animals supplied with the vitamin E-deficient diet for as long as 6 months.

4. Lipoperoxides in adipose tissue varied greatly in amount in animals undergoing tocopherol depletion with the diet used in these experiments. Appreciable amounts were present at three weeks, and maximal levels were reached at 7 to 9 weeks. Peroxides were usually at low levels or absent in animals maintained with the vitamin E-deficient diet for a year or more.

5. The development of an increased rate of postmortem autolysis in the kidney was always preceded by a reduction in renal tocopherol level and the appearance of peroxidation in the adipose tissue.

6. These observations are consistent with the view that an abnormal state of intracellular lipids may contribute to the increased rate of postmortem autolysis which occurs in the kidneys of these animals.

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Compositions of Skeletal Muscle Lipids of Rats Fed Diets Containing Various Oils [1,2]

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The requirement for vitamin E in animals (Briggs et al., '56; Century et al., '59; Century and Horwitt, '59, '60; Machlin and Gordon, '60) and in man (Horwitt, '60; Horwitt et al., '61) has been related to the polyunsaturated fatty acids either ingested or accumulated in tissues or both. Nutritional dystrophy and concomitant increases in urinary creatine excretion were most rapidly and readily produced in rats by feeding tocopherol-deficient diets containing high levels of polyunsaturated fatty acid (PUFA) — containing lipids, such as those from 7% of cod liver oil, 7% of linseed oil, or 15% of corn oil. Diets containing these oils, from which most of the tocopherols had been removed, produced an elevated creatinuria in 9 to 15 weeks, whereas similar diets containing 15% of coconut oil or 0.2% of corn oil produced no signs of tocopherol deficiency up to 29 weeks (Century and Horwitt, '60). It was therefore pertinent to examine the effects of the ingestion of various oils upon the fatty acid compositions of rat skeletal muscle lipids. Accordingly, diets containing oils of widely differing compositions were fed to rats receiving adequate supplements of vitamin E, in order to evaluate the role of incorporation and storage of various fatty acids into normal skeletal muscle as a factor in determining the potential sensitivity of muscle tissue to tocopherol deprivation.

EXPERIMENTAL

Male weanling Sprague-Dawley rats were supplied with synthetic diets containing 0.2% of corn oil, 15% of coconut oil, 15% of corn oil, or 7% of cod liver oil (table 1). These were representative of (1) low fat, (2) high saturated fat, (3) high essential fatty acid, and (4) high nonessential PUFA, but low essential fatty acid-containing diets, respectively. The oils were obtained from commercial sources and analyzed for fatty acid compositions by gas-liquid chromatography. Lipids and water-soluble vitamins were mixed into stock ingredients of the diets every two or three days and kept refrigerated. Feed cups were emptied and refilled daily. Vitamins A (2500 I.U.) and D (360 I.U.) were given orally every week, and 12.5 mg (17.0 I.U.) of *d*-α-tocopheryl acetate supplements were given weekly in two oral doses. Skeletal muscle samples from weanlings and from 6- and 21-week experimental rats were weighed and homogenized in methylal-methanol (4:1) with 0.01% of α-tocopherol as an added antioxidant, and methyl esters were prepared from the extracted lipids by the method of Stoffel et al. ('59), with modifications. Fatty acid compositions were determined by gas-liquid chromatography on Celite columns with ethyleneglycolsuccinate polyester as the liquid phase, using tritium ionization cell detectors. Identifications of chain length and the degree of unsaturation of specific fatty acids were

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[2] A preliminary report of this work was given at the 1961 meeting of the Federation of American Societies for Experimental Biology, Atlantic City: Century, B., L. A. Witting, C. C. Harvey and M. K. Horwitt 1961 Fatty acid compositions in skeletal muscle of rats on diets containing various lipids. Federation Proc., *19*: 367.

TABLE 1

Basal diets with isocalorically substituted lipids

Ingredient[1]	0.2% Corn oil	15% Coconut oil	15% Corn oil	7% Cod liver oil
	gm	*gm*	*gm*	*gm*
Casein (vitamin-test)	25	25	25	25
Salts (USP 14)	4	4	4	4
Dextrose	48	30	30	39
Cornstarch	42	26	26	33.2
Lipid[2]	0.24	15	15	7.8
Choline dihydrogen citrate	0.33	0.33	0.33	0.33
Vitamin mix[3]	0.33	0.33	0.33	0.33

[1] For clarity in showing isocaloric substitutions, ingredients are given in parts rather than percentages.

[2] Major components of dietary oils: (in per cent) Coconut oil, lauric, 28; myristic, 28; palmitic, 17; stearic, 6; oleic, 14; and linoleic, 4: Corn oil, palmitic, 8; oleic, 30; and linoleic, 54: Cod liver oil, palmitic, 8; palmitoleic, 18; eicosaenoic, 13; arachidonic (isomer), 7; eicosapentaenoic, 15; and docosahexaenoic, 12.

[3] Vitamin mix contains: (in milligrams) *i*-inositol, 11.1; p-aminobenzoic acid, 11.1; D-calcium pantothenate, 6.2; 2-methyl-1-4-naphthoquinone, 5.0; niacin, 10.0; thiamine·HCl, 2.2; pyridoxine·HCl, 2.2; riboflavin, 2.2; folic acid, 0.2; biotin, 45 μg; vitamin B_{12}, 3 μg; and starch to 0.33 gm.

primarily based upon considerations of the data of Farquhar et al. ('59), Woodford and van Gent ('60), and some further identification procedures as described by Witting et al. ('61).

RESULTS

It is apparent that dietary lipids greatly influenced the composition of fatty acids in skeletal muscle of rats (table 2). Higher percentages of linoleic ($P < 0.01$) and arachidonic ($P < 0.005$) acids and lower levels of oleic acid ($P < 0.02$) were seen in samples from rats fed 15% of corn oil after 6 and 21 weeks, as compared with results from weanlings. Linoleic acid was lower in samples from rats fed 0.2% of corn oil ($P < 0.001$), 15% of coconut oil ($P < 0.001$), or 7% of cod liver oil ($P << 0.001$), whereas palmitoleic acid was increased in all three groups whose diets were low in linoleic acid content ($P << 0.001$). An extremely low percentage of arachidonic acid was observed in muscle lipids from rats receiving 7% of cod liver oil ($P << 0.001$). Samples from this group were further characterized by having no detectable docosahexaenoic acid (22:6), significantly higher levels of docosapentaenoic acid (22:5) ($P < 0.005$), markedly higher percentages of Δ 7,10,13,16,19-docosapentaenoic acid ($P << 0.001$), and by the appearance of an isomer of eicosapentaenoic acid (20:5) other than the one seen in animals on the other three diets (20:5 Δ 5,8,11,14,17). A higher level of an eicosatrienoic acid (20:3) isomer was also observed, which might possibly have its double bonds either at the 7,10 and 13 or at the 8,11, and 14 positions. The appearance of significant amounts of 5,8,11-eicosatrienoic acid in the 0.2% corn oil and 15% coconut oil groups is characteristic in animals low in essential fatty acids (Mead and Slaton, '56). Lauric and myristic acids remained elevated only in samples from rats fed 15% of coconut oil, apparently due to the high levels of these fatty acids in coconut oil.

No signs of essential fatty acid deficiency were observed in rats fed 0.2% of corn oil. In contrast, another group of rats maintained with a fat-free diet for 8 weeks showed signs of acrodynia and yielded skeletal muscle lipids having the following components: palmitic acid, 25.7%; palmitoleic acid, 11.8%; oleic acid, 42.0%; linoleic acid, 1.0%; and arachidonic acid, 1.6%. These data indicated that the latter animals were severely deficient in essential fatty acids and had considerable compensatory increases in the major monoenes.

No significant differences were observed in the growth rates of the animals, which were similar to those published previously (Century and Horwitt, '60).

TABLE 2

Fatty acid compositions of lipids from skeletal muscle of rats fed various oil diets

Fatty acid	Weanlings	6 Weeks				21 Weeks			
		0.2% Corn oil	15% Coconut oil	15% Corn oil	7% Cod liver oil	0.2% Corn oil	15% Coconut oil	15% Corn oil	7% Cod liver oil
No. of samples	5	5	5	5	5	5	5	5	3
	%[1]	%	%	%	%	%	%	%	%
12:0 Lauric	3.6 ± 0.4[2]	0.3 ± 0.2	5.5 ± 0.4	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	4.9 ± 0.4	0.2 ± 0.1	0.2 ± 0.1
14:0 Myristic	6.0 ± 0.4	1.7 ± 0.2	6.1 ± 0.4	0.7 ± 0.1	2.4 ± 0.1	1.5 ± 0.2	5.5 ± 0.5	0.5 ± 0.1	2.1 ± 0.05
16:0 Palmitic	16.5 ± 1.0	17.1 ± 3.8	17.1 ± 0.2	15.0 ± 0.8	15.8 ± 0.9	16.6 ± 1.0	13.9 ± 0.7	13.4 ± 0.2	18.3 ± 2.6
18:0 Stearic	11.7 ± 1.1	13.2 ± 0.8	10.6 ± 0.3	11.7 ± 0.6	9.9 ± 0.6	11.2 ± 0.5	11.6 ± 0.8	11.4 ± 0.3	9.6 ± 0.3
20:0 Arachidic	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.05	0.2 ± 0.05	nil	0.5 ± 0.2	0.2 ± 0.05	0.2 ± 0.02	nil
24:0 Lignoceric	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.2	2.0 ± 0.6	0.4 ± 0.02	0.6 ± 0.2	0.8 ± 0.1	1.4 ± 0.1	0.3 ± 0.1
16: aldehyde	0.8 ± 0.3	2.1 ± 0.5	1.3 ± 0.1	1.7 ± 0.1	1.5 ± 0.3	2.6 ± 0.3	2.0 ± 0.2	1.7 ± 0.1	2.0 ± 0.1
18: aldehyde	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
16:1 Palmitoleic	2.2 ± 0.3	8.0 ± 1.1	6.8 ± 0.4	1.6 ± 0.5	8.2 ± 0.5	9.0 ± 0.6	6.4 ± 0.6	1.1 ± 0.2	7.7 ± 0.3
18:1 Oleic	24.0 ± 1.6	24.7 ± 1.4	24.0 ± 1.2	16.7 ± 1.7	21.7 ± 1.3	22.0 ± 2.3	20.3 ± 0.4	17.6 ± 1.1	23.5 ± 2.4
20:1	1.7 ± 0.5	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	5.1 ± 0.8	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	2.8 ± 0.05
18:2	18.1 ± 0.4	9.2 ± 0.8	10.1 ± 0.7	26.1 ± 2.0	5.8 ± 0.4	12.0 ± 0.8	12.6 ± 0.7	23.8 ± 0.8	6.3 ± 0.7
20:2	0.6 ± 0.1	0.4 ± 0.1	0.2 ± 0.05	0.6 ± 0.2	nil	0.2 ± 0.1	0.1 ± 0.05	0.4 ± 0.1	nil
22:2	0.1 ± 0.05	0.2 ± 0.1	0.2 ± 0.05	0.2 ± 0.05	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:3 ($\Delta^{5,8,11}$)	nil	4.1 ± 0.8	2.7 ± 0.4	nil	0.4 ± 0.1	2.9 ± 0.3	1.4 ± 0.1	nil	0.4 ± 0.05
20:3	0.8 ± 0.1	1.2 ± 0.3	1.0 ± 0.1	0.6 ± 0.2	2.9 ± 0.3	1.3 ± 0.2	0.9 ± 0.1	0.5 ± 0.05	2.2 ± 0.3
20:4 Arachidonic	5.8 ± 0.9	7.7 ± 0.8	7.8 ± 0.6	14.9 ± 1.8	0.6 ± 0.1	11.3 ± 1.5	10.0 ± 0.5	15.9 ± 1.0	0.3 ± 0.05
20:5	nil	nil	nil	nil	3.9 ± 0.2	nil	nil	nil	2.9 ± 0.7
22:5[3] ($\Delta^{5,8,11,14,17}$)	0.7 ± 0.2	1.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	nil	0.6 ± 0.4	0.4 ± 0.05	0.4 ± 0.05	nil
22:5 •	0.3 ± 0.02	0.5 ± 0.1	0.7 ± 0.1	1.6 ± 0.4	2.5 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	2.2 ± 0.2	2.0 ± 0.5
22:5[3] ($\Delta^{7,10,13,16,19}$)	1.5 ± 0.3	0.5 ± 0.2	0.6 ± 0.1	1.0 ± 0.2	16.4 ± 0.8	0.8 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	17.7 ± 2.6
22:6	1.8 ± 0.3	2.3 ± 0.3	1.6 ± 0.5	2.5 ± 0.6	nil	3.5 ± 1.0	4.5 ± 0.4	5.3 ± 0.6	nil

[1] This is percentage of total methyl esters of fatty acids determined by gas chromatography; some minor components have been omitted from this table.

[2] Standard error.

[3] Isomers normally found in cod liver oil.

TABLE 3

Fatty acids in skeletal muscle in order of increasing unsaturation, and estimated peroxidizability ratings of skeletal muscle lipids

Fatty acid classification	Weanlings	6 Weeks				21 Weeks			
		0.2% Corn oil	15% Coconut oil	15% Corn oil	7% Cod liver oil	0.2% Corn oil	15% Coconut oil	15% Corn oil	7% Cod liver oil
No. of samples	5	5	5	5	5	4	5	5	3
	%	%	%	%	%	%	%	%	%
Saturated	39.9	35.8	41.9	32.0	30.6	33.7	39.7	29.6	33.3
Monoene	27.9	33.2	31.3	18.8	35.0	31.6	27.3	19.3	34.0
Diene	18.8	9.8	10.5	26.9	5.9	12.4	12.9	24.5	6.6
Triene	0.8	5.3	3.7	0.6	3.3	4.2	2.3	0.5	2.6
Tetraene	5.8	7.7	7.8	14.9	0.6	11.3	10.0	15.9	0.3
Pentaene	2.5	2.5	1.7	3.2	22.8	2.6	2.2	3.8	22.6
Hexaene	1.8	2.3	1.6	2.5	nil	3.5	4.5	5.3	nil
Peroxidizability estimate	77 ± 10	90 ± 8	75 ± 9	135 ± 16	197 ± 8	115 ± 19	112 ± 5	161 ± 12	194 ± 31
P			< 0.02	< 0.01			< 0.01		
			< 0.05				< 0.01		
			< 0.001				0.05 < P < 0.1		
							0.05 < P < 0.1		

DISCUSSION

It is apparent that lipids from skeletal muscle of rats fed 15% of corn oil or 7% of cod liver oil contained more of the highly polyunsaturated fatty acids which are expected to be more easily peroxidized in the absence of *in vivo* protective reducing agents, such as tocopherol, as compared with similar muscle lipids obtained from the 0.2% corn oil or 15% coconut oil experimental groups (tables 2 and 3). It has been generally assumed that the rate of autoxidation of PUFA is related to the number of double bonds in the molecule (Holman, '54; Horwitt et al., '61). A tentative approximation of the relative peroxidizability of the muscle lipids might be made by multiplying the diene percentages by one, the trienes by two, the tetraenes by 4, and the pentaenes and hexaenes by 8. Using such an estimate, significantly higher values were obtained for the muscle lipids from animals in the 15% corn oil and 7% cod liver oil groups after 6 weeks (table 3). These differences in estimated peroxidizability became statistically less significant after 21 weeks, partly due to increased deposition of PUFA in the muscles of animals of the 0.2% corn oil and the 15% coconut oil groups. Apparently, there is a tendency for skeletal muscles to accumulate PUFA at a slow rate when the rats are fed diets low in PUFA.

The ingestion of 7% of cod liver oil or 15% of corn oil, which resulted in high creatinuria and dystrophy in vitamin E-deficient rats (Century and Horwitt, '59), also resulted in higher percentages of the more highly unsaturated fatty acids in muscle lipids, whereas the reverse was true when diets of 0.2% of corn oil or 15% of coconut oil were fed. This suggests at least a correlation between the amount and degree of polyunsaturation of the muscle lipids and the occurrence of nutritional dystrophy in the vitamin E-deficient rat (Century and Horwitt, '60).

Examination of table 2 shows increasing percentages of linoleic and arachidonic acids between the 6 and 21 weeks' samples from the 0.2% corn oil and 15% coconut oil groups, and suggests increasing percentages of docosapentaenes and hexaenes after 21 weeks in muscle lipids from the rats fed 0.2 and 15% of corn oil and 15% of coconut oil. The suggestion of a greater degree of unsaturation of the muscle lipids as the animal gets older makes it necessary to evaluate by additional experimentation whether with a diet low in PUFA one can with sufficient age accumulate enough PUFA in the tissues to approach in an older animal the levels of PUFA one finds in the tissues of a young animal which has been ingesting high levels of PUFA.

SUMMARY

Rats fed diets containing 0.2% of corn oil, 15% of coconut oil, 15% of corn oil, or 7% of cod liver oil for 6 and 21 weeks showed considerable differences in fatty acid compositions of skeletal muscle lipids. Samples from the 15% corn oil group were characterized by high percentages of linoleic and arachidonic acids with compensatory decreases in palmitoleic and oleic acids, whereas those from the 7% cod liver oil group had high levels of pentaenes. A correlation appears to exist between the increased polyunsaturated fatty acid percentages in muscle lipids and the dietary conditions producing nutritional dystrophy in the vitamin E-deficient rat.

A slight increase was observed in the degree of unsaturation of muscle fatty acids in rats fed 0.2 or 15% of corn oil or 15% of coconut oil for 21 weeks, as compared with 6 experimental weeks.

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Protective Action of Bile Acids in Experimental Thyrotoxicosis

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The effects of thyroid hormones on cholesterol and bile acid metabolism have been widely studied. There have been but few studies of the reverse relationship. Doisy and Lardy ('57) studied steroidal compounds as peripheral antagonists of the calorigenic actions of the thyroid hormone. Numerous investigators have reported that liver residue and other crude materials in the diet protect rats against chronic administration of thyroxine without measurably reducing the basal metabolic rates (Overby et al., '59a, b, c,'60). Such protection has not yet been reproduced with known nutrients.

Ershoff and Marx ('48), and Marx et al. ('48) reported the favorable effect of dietary cholesterol on experimental thyrotoxicosis. Others have similarly tested steroidal compounds (Westerfeld and Richert, '52; Stevens and Henderson, '58; Dryden et al., '60).[1] In attempting to identify the active components of liver we found that solvent extraction of liver residue gave an active lipid extract without loss of activity in the defatted residue (Overby et al., '59b). The lipid extract was about 75% neutral fat, 9% cholesterol, 8% phospholipids and 8% bile acid and unidentified products. Representative examples of these classes of lipids were then tested separately. The experiments herein report the specificity of the protection of bile acids in experimental thyrotoxicosis and give some information on possible mechanisms of action.

EXPERIMENTAL

The experimental techniques including animals and diets have been described previously (Overby et al., '59a). Male, 21-day-old Sprague-Dawley rats were used in groups of 10. They were fed basal diet 14 composed in percentage of casein, 30; sucrose, 57.5; cottonseed oil, 5; salts, 4; cellulose and agar, 3.25; choline chloride, 0.1; and vitamins, 0.15. In each experiment three control diets were used: (1) the basal diet; (2) the negative control (basal plus 0.35% of iodinated casein);[2] and (3) the standard (negative control plus 10% of defatted liver residue). The test groups received the negative control diet with the test material replacing an equal weight of sucrose. The test material was also assayed in the presence of liver residue.

Weight gains and survival at 5 weeks were the measures of protection. Liver residue promoted both. Activity of the test materials could be interpreted best in terms of their own controls. Therefore to rate objectively the active materials and to compare different experiments, a relative potency was calculated with the standard liver residue as 100 and the negative control zero (Overby and Fredrickson, '60). The nonthyrotoxic basal diet gave values of 120 to 160.

RESULTS

In table 1 are shown the results with several bile products and bile acids. The relative potencies are from different experiments but refer to the same standard liver residue. Weight gains and survivals are omitted for conciseness. In group 1 different levels of 4 bile acids and cholesterol are compared. Lithocholic acid, hyodeoxycholic acid and cholesterol were inactive in these tests and appeared to adversely affect the thyrotoxic rat. Deoxycholic acid

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[1] Page, A. C., F. R. Koniusky, Jr., D. E. Wolf, P. Aldrich and K. Folkers 1956 Factors in liver reversing thyroid stress in rats. Federation Proc., *15:* 568 (abstract).

[2] Protamone, Cerophyl Laboratories, Kansas City, Missouri.

TABLE 1

Comparative antithyrotoxic activity of bile acids and bile products

Group	Bile product	% in diet	Relative potency at 5 weeks[1]		
			Basal	Test materials	
				Without liver residue	With 10% of liver residue
	Comparison of bile acids and cholesterol				
1 a	Cholic acid	0.1	130 ± 4.5[3]	31 ± 10.5	116 ± 11.7
b	Deoxycholic acid	0.1		57 ± 4.6	113 ± 11.7
c	Hyodeoxycholic acid	0.1		−8 ± 9.4	59 ± 9.5
d	Cholic acid	0.2		44 ± 5.5	105 ± 6.6
e	Deoxycholic acid	0.2		71 ± 4.5	126 ± 4.5
f	Hyodeoxycholic acid	0.2		3 ± 9.4	
g	Lithocholic acid	0.2		−22 ± 9.4	
h	Cholesterol	0.2		17 ± 10.5	100 ± 8.0
i	Cholesterol	0.4		−10 ± 10.5	78 ± 11.7
	Comparison of different levels of deoxycholic acid				
2 a	Deoxycholic acid	0.05	119 ± 6.1	32 ± 3.4	122 ± 5.0
b	Deoxycholic acid	0.10		57 ± 3.6	119 ± 4.9
c	Deoxycholic acid	0.20		82 ± 7.6	120 ± 4.9
d	Deoxycholic acid	0.40		79 ± 2.7	121 ± 1.9
e	Deoxycholic acid	0.80		84 ± 4.6	112 ± 5.1
	Comparison of crude bile products				
3 a	Ox bile powder[2]	0.1	128 ± 5.9	28 ± 11.2	100 ± 15.8
b	Ox bile powder	0.2		54 ± 8.2	103 ± 5.0
c	Ox bile powder	0.4		66 ± 6.7	104 ± 9.1
d	Ox bile powder	0.8		71 ± 11.2	117 ± 12.0
e	Ox bile powder	1.6		72 ± 9.1	112 ± 15.8
f	Hog bile powder[2]	0.4		63 ± 11.2	103 ± 15.8
g	Deoxycholic acid + ox bile powder	0.2 0.8		57 ± 11.2	121 ± 15.8
	Combinations of bile products				
4 a	Cholic acid + deoxycholic acid	0.1 0.1	158 ± 3.3	51 ± 7.6	131 ± 7.6
b	Cholic acid + deoxycholic acid + cholesterol	0.2 0.2 0.2		70 ± 10.8	137 ± 10.8
c	Cholic acid + deoxycholic acid + cholesterol	0.2 0.2 0.4		78 ± 10.8	143 ± 10.8
d	Cholic acid + cholesterol	0.2 0.2		48 ± 10.8	91 ± 10.8

[1] By definition negative control = 0; standard liver residue = 100.
[2] The dried alcohol-soluble portion of crude bile.
[3] The mean standard error of the mean relative potency determined from a pooled variance.

appeared to give better protection than cholic acid. The 0.2% level with liver residue gave almost complete protection: relative potency of 126 versus 130 for the nonthyrotoxic basal.

The second test (group 2, table 1) measured the response to different levels of deoxycholic acid. Maximum response without liver residue was obtained with 0.2% deoxycholic acid. Food intake was reduced at levels above 0.8%. As little as 0.05%, slightly active alone, gave complete protection with liver residue in this experiment. Usually, however, the combination of bile acid and liver residue was a few points below the basal potency.

For group 3, the activities of the alcohol-soluble fraction of ox bile and hog bile are shown (table 1). Maximum response was obtained with 0.8% of ox bile powder. The diet was unpalatable at higher levels. The ox bile powder contained cholic and

deoxycholic acids. Hog bile powder, containing a high percentage of hyodeoxycholic acid, was equal to ox bile at the 0.4% level, although hyodeoxycholic acid alone was inactive. The low levels of bile powders, slightly active alone, did not give enhanced potency with liver residue.

In another series of experiments (group 4, table 1) combinations of cholic acid, deoxycholic acid and cholesterol were tested. The results suggested that activity could be accounted for by the deoxycholic acid in the mixture. As observed in group 1, cholic acid maximum potency was about 50.

The above experiments indicated that the response to deoxycholic acid at 0.2% of the diet could not be duplicated or improved by the other bile products tested. Therefore, this level of deoxycholic acid was used in 34 experiments to study fractionation of defatted liver residue for activity different from the bile acid response. In table 2 are shown the results of the groups receiving deoxycholic acid with and without the standard liver residue. The bile acid was 61% as active as the standard. The combination of bile acid and liver residue gave growth and survival approaching the nonthyrotoxic basal. In no case was it equal. The mean weight gains were 197 and 204 in this series of experiments. The fractionation studies yielded material (believed to be devoid of protein, neutral fat, cholesterol and bile acids) 10 times as active as the defatted liver residue.[3]

Another parameter for measuring antithyrotoxic activity is the inhibition of adrenal hypertrophy. In one experiment the adrenals were removed and weighed. The groups listed in decreasing order of adrenal weight per 100 gm of body weight are shown in table 3. Actually the body weights and survivals follow the same order with but few exceptions. Adrenal hypertrophy was inhibited most (but not prevented) by the combinations of bile products and liver residue. Deoxycholic acid and ox bile were most active in this respect. Liver residue alone was intermediate in activity, followed by the bile products alone. Cholesterol and cholic acid alone appeared to have little effect in preventing adrenal hypertrophy. The response to all combinations appeared to be additive.

Bile acids play key roles in the absorption of various nutrients. In the foregoing experiments the thyroactive substance was administered orally simultaneously with the bile acid. It was possible that the favorable response to bile acids merely reflected decreased absorption or other inactivation of the iodinated casein. The results of an experiment comparing responses to oral iodinated casein and injected DL-thyroxine are shown in table 4. Both deoxycholic acid and liver residue were active when tested against injected thyroxine. The degree of thyrotoxicosis was not well controlled in the injected groups; hence they could not be compared quantitatively with the iodinated casein groups. In several other experiments surface active agents, such as polysorbate 80

[3] Preparation of the materials will be reported in subsequent publications.

TABLE 2

Comparative activity of liver residue and deoxycholic acid in 34 experiments

Dietary treatment	5-Week results		
	Weight gain	Survival	Relative potency
	gm	%	
Basal diet 14	204 ± 3.3[1]	100	134 ± 6.6
Negative control[2]	132 ± 3.1	50 ± 5.2	0
Standard (10% of liver residue)	184 ± 2.9	95 ± 1.5	100
Deoxycholic acid (0.2%)	164 ± 3.0	86 ± 3.2	61 ± 5.6
Deoxycholic acid (0.2%) + liver residue (10%)	197 ± 3.3	98 ± 0.2	132 ± 5.7

[1] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

[2] This and subsequent groups contained 0.35% iodinated casein: Protamone, Cerophyl Laboratories, Kansas City, Missouri.

TABLE 3

Effect of bile acids, cholesterol and liver residue on adrenal hypertrophy in thyrotoxic rats

Dietary treatment[1]			5-Week results		
Group	Supplement		Surviving	Body weight	Adrenals weight
		%	%	*gm*	*mg/100 gm body*
1	None (non-thyrotoxic basal)		100	277	17 ± 0.93[2]
2	Deoxycholic acid + liver residue	0.2 10	100	267	20 ± 0.95
3	Ox bile powder + liver residue	0.2 10	100	260	21 ± 1.05
4	Cholic acid + liver residue	0.2 10	100	253	25 ± 1.18
5	Cholesterol + liver residue	0.2 10	70	229	26 ± 1.74
6	Liver residue	10	100	225	30 ± 2.21
7	Cholesterol + cholic acid	0.2 0.2	50	229	31 ± 3.00
8	Ox bile powder	0.2	40	234	33 ± 6.15
9	Deoxycholic acid	0.2	60	215	35 ± 3.38
10	Cholic acid	0.2	40	201	43 ± 0.76
11	Cholesterol	0.2	30	193	44 ± 2.67
12	None (negative control)[3]		0	165–195	45–50

[1] All groups except no. 1 contained 0.35% iodinated casein.

[2] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

[3] There were no surviving animals in this group. A range of values found in other similar experiments is given.

TABLE 4

Comparison of antithyrotoxic activity of liver residue and deoxycholic acid in iodinated casein-fed and thyroxine-injected rats

Diet treatment	5-Week results			
	Iodinated casein[1]		DL-Thyroxine[2]	
	Weight gain	Survival	Weight gain	Survival
	gm	%	*gm*	%
Basal no. 14	233 ± 5.6[3]	100	233 ± 5.6	100
Negative control	—	0	144 —	10
Liver residue (10%)	181 ± 4.6	100	163 ± 4.9	80
Deoxycholic acid (0.2%)	176 ± 4.8	50	177 ± 4.7	90

[1] All groups except the basal recieved iodinated casein at 0.35% of the diet.

[2] Subcutaneously injected in all groups except the basal. Each rat received three times each week a solution containing the following micrograms of DL-thyroxine: first week, 66; second week, 500; third week, 1000; 4th week, 1150; 5th week, 1500.

[3] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

and lecithin, had no antithyrotoxic activity either with or without liver residue.

Previous experiments (Overby et al., '59b) showed that unsaturated fats exerted a favorable antithyrotoxic effect. It was possible that the deoxycholic acid effect was a result of better utilization of the dietary cottonseed oil. An experiment was carried out to compare the relative activity of a fraction from liver residue[4] (free of neutral fat, cholesterol and bile acids) and deoxycholic in a ration containing 0.5% of hydrogenated coconut oil[5] as the only fat. Results are shown in table 5. Thyrotoxicity was increased, and the protective effect of deoxycholic acid was of borderline significance with the fat-deficient diet.

[4] See footnote 3.

[5] Hydrol, Durkee's Famous Foods, Chicago.

TABLE 5

Comparison of antithyrotoxic activity of deoxycholic acid and a liver residue fraction in diets with 5% of cottonseed oil and 0.5% of hydrogenated coconut oil

Dietary treatment	5-Week results			
	5% Cottonseed oil		0.5% Hydrogenated coconut oil	
	Weight gain	Survival	Weight gain	Survival
	gm	*%*	*gm*	*%*
Nonthyrotoxic basal	220 ± 3.1[2]	100	201 ± 5.7	100
Negative control[1]	154 ± 7.1	20	83 ± 3.3	40
Liver residue fraction (1.85%)	215 ± 5.1	90	150 ± 4.5	100
Deoxycholic acid (0.2%)	191 ± 8.4	80	100 ± 4.4	60

[1] This and subsequent groups contained 0.35% iodinated casein; Protamone, Cerophyl Laboratories, Kansas City, Missouri.

[2] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

TABLE 6

Antithyrotoxic activity of deoxycholic acid in rats receiving sulfasuxidine and neomycin

Diet treatment	5-Week results			
	Normal diet		Chemotherapeutic diet[1]	
	Weight gain	Survival	Weight gain	Survival
	gm	*%*	*gm*	*%*
Basal	216 ± 5.7[2]	100	202 ± 4.8	100
Negative control[3]	86 —	10	88 —	10
Liver residue[3] (10%)	168 ± 4.7	90	—	—
Deoxycholic acid[3] (0.2%)	145 ± 5.9	80	139 ± 6.3	70

[1] 1.5% of sulfasuxidine and 0.15% of neomycin sulfate.

[2] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

[3] Diet contained 0.35% of iodinated casein.

The intestinal flora play a large part in the over-all metabolism of bile acids. It was possible that the observed antithyrotoxic effects of deoxycholic acid were actually indirect responses via the intestinal flora. In table 6 are shown the results of studies done with rats in which the intestinal flora were severely altered by feeding a diet containing 1.5% of sulfasuxidine and 0.15% of neomycin sulfate. This regimen caused slight diarrhea and inhibition of growth, but did not alter the expected thyrotoxic protection of deoxycholic acid. Liver residue was not tested with the chemotherapeutic diet in this particular experiment, but it was active in other similar studies. Thus it appeared that the antithyrotoxic activity of deoxycholic acid, and liver residue, was not mediated via the intestinal flora.

DISCUSSION

Bile acids are normally classed as end products of cholesterol metabolism. They are necessary components for emulsification and absorption of dietary substances, but an exogenous requirement is noted only in cases of biliary obstruction or malfunction. Animal species, intestinal bacteria, and thyroid hormone are influential factors in the over-all metabolism of cholesterol and bile acids. The bile acids found in tissues, fluids and excreta are thus the net results of action mediated by several factors. Studies are difficult because of this multiplicity of influencing factors and the entero-hepatic recirculation in normal animals.

Several previous studies have shown some effects of steroidal compounds on thyroxine metabolism. Marx et al. ('48) observed that a diet containing 1% of cholesterol and 0.5% of bile salts prolonged the survival of rats receiving desiccated thyroid. Ershoff and Marx ('48) repeated these experiments without bile salts and reported a survival effect due to cholesterol. Westerfield and Richert ('52) observed that cho-

lesterol had no effect on hyperthyroid rats fed iodinated casein. Page et al.[6] reported that cholesterol partially reversed thyroid stress in rats. Several pure bile acids and other steroids were also effective. Stevens and Henderson ('58) noted that cholesterol alone had no growth-promoting activity in hyperthyroid rats, but had a small effect when fed with 5.5% of corn oil. Dryden et al. ('60) also tested combinations of cholesterol and increased fat. Singly they were inactive for promoting growth of hyperthyroid rats; in combination they gave large increases in growth.

The metabolism of cholesterol is clearly changed by the thyroid hormone (Byers, '58). Excretion is markedly increased in hyperthyroidism and decreased in hypothyroidism (Eriksson, '57). The various reports summarized above imply conflicting results as to the effect of dietary cholesterol in experimental hyperthyroidism. Under our experimental conditions little or no protective effects were observed for cholesterol. Certain bile acids, end products of cholesterol metabolism, had protective actions. Clearly, the most active bile acid tested was deoxycholic acid. In the rat, it is derived from cholic acid by bacterial action (Lindstedt and Samuelson, '59), and is largely reconverted to cholic acid by the liver. In normal rat excreta deoxycholic acid is the major bile acid. In the bile, cholic and chenodeoxycholic acids predominate in a ratio of about 8 to 12. The total output in the bile is not quantitatively changed in hyperthyroid rats; however, the ratio of cholic to chenodeoxycholic approaches one-to-one (Eriksson, '57). In the hypothyroid state, formation of both acids is diminished. None of the cholic acid metabolites found in normal rat feces are found in germfree rats or those treated chemotherapeutically to sterilize the gut (Gustafsson et al., '57).

Thus it is unexpected that deoxycholic acid, a bacterial product of cholic acid in that rat, is so effective in protecting against thyrotoxicosis. Its relation to fractions of hog liver is also not clear. Hyodeoxycholic acid, a bacterial product of hyocholic acid in swine, was not antithyrotoxic in rats. Furthermore, it is possible to obtain fractions from hog liver, free of bile acids, equal in response to the crude liver residue. The almost complete restoration of growth by the combination of deoxycholic acid and liver residue could not be reproduced with either alone. The crude ox bile products contained mostly cholic and deoxycholic acids. The protective effects of these materials could be accounted for by the bile acid content, rather than some unidentified material observed in the bile. The crude hog bile, although active, probably contained mostly hyocholic and hyodeoxycholic acids, inactive bile acids. The relative activity of beef and pork liver residue is uncertain (Stevens and Henderson, '58; Dryden et al., '60). Both crude products have antithyrotoxic activity, although they contain different dihydroxycholanic acids: deoxycholic in beef liver and hydodeoxycholic in pork liver.

There are several speculations that might account for the antithyrotoxic effect of deoxycholic acid:

1. It may be an essential metabolite or specific precusor for some essential metabolite, hormone, or nutrient required for thyrotoxic rats.
2. It may favorably influence the bacterial activity in the intestine.
3. It may prevent the absorption, inactivate, or increase the elimination of the thyroactive compound.
4. It may indirectly improve the absorption or utilization of some essential material supplied by the diet or by synthesis by the animal. Deoxycholic acid has the unique character of forming remarkably stable coordination compounds (choleic acids), giving to substances considerably modified properties of solubility and diffusibility.

The experiments reported herein would tend to rule out the second and third possibility. More specific biochemical tests are required to establish the mechanism of action and the relations to the nonbile acid protective principles found in hog liver.

SUMMARY

Deoxycholic acid protected rats from experimental thyrotoxicosis. Hyodeoxycholic acid, lithocholic acid and cholesterol were inactive. Cholic acid was slightly active. Fractions from liver, containing neither

[6] See footnote 1.

neutral lipids, bile acids nor cholesterol, also protected rats from chronic thyrotoxicosis. The maximum response from deoxycholic acid was about 60% of the maximum obtained with liver fractions. The combination of deoxycholic acid and liver gave almost complete protection. The substances were active with oral or injected thyroxine, and in rats with chemotherapeutically-sterilized intestinal tracts.

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Calcium Utilization and Skeletal Development in Chicks as Influenced by Parental Dietary Ascorbic Acid[1]

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Earlier work from this laboratory (Thornton et al., '59) with chicks indicated that the presence of vitamin C in the parents' diet influenced the skeletal retention of Ca^{45} by rachitic progeny. In this work it was shown that the amount of skeletal deposition of Ca^{45} was highly correlated with the quantity of isotope passing from the nonabsorptive to the absorptive area of the bowel in both control and rachitic chicks from parents fed ascorbic acid-supplemented diets. Control progeny from parents not given vitamin C reacted similarly, whereas rachitic progeny did not show this correlation.

The mode of action of vitamin D is not yet clear, despite a great volume of work which has been devoted to the problem as reviewed by Nicolaysen and Eeg-Larsen ('53). Recent work (Schachter and Rosen, '59) strongly supports the concept that vitamin D is essential for active transport of calcium at the intestinal wall level. Other research (Keane et al., '56; Migicovsky and Jamieson, '55) also indicated that vitamin D was necessary for optimal absorption. The results with rachitic chicks from control parents (Thornton et al., '59) also support the theory that a deficiency of vitamin D_3 decreases the intestinal absorption of calcium. But data obtained in this same study with rachitic chicks from parents given dietary vitamin C indicated that intestinal absorption of calcium was not affected by the absence of vitamin D_3. Rachitic symptoms in such chicks were clearly as severe as those observed in chicks from parents not given vitamin C. Thus it seemed that merely getting calcium to the skeleton was not effective for the prevention of rickets. Such results indicate that vitamin D_3 is influential in the prevention of rickets by playing a role in normal bone growth and metabolism. The recent observation by Belanger and Migicovsky ('60) that vitamin D promoted maturation of cartilage cells as well as being involved with calcium in the maturation of subepiphyseal bone cells and matrix strongly supports the concept that this vitamin is involved in bone growth.

In the present studies the influence of parental ascorbic acid was further studied with observations on skeletal Ca^{45} being extended to 24 hours after intestinal injection.

EXPERIMENTAL

Two studies were conducted to observe the influence of parental dietary ascorbic acid on the uptake and biological turnover of Ca^{45} in the young chick given control and rachitogenic diets. New Hampshire × Delaware cross chicks were used in each study. In the first, the chicks were produced from parents given a control breeding diet and the same diet supplemented with 20 or 40 mg of ascorbic acid per pound of ration at one day of age. These chicks were supplied with either a control diet (Thornton et al., '59) or this diet with the vitamin D_3 and steamed bone meal removed. Chicks were separated on a basis of the presence or absence of vitamin C in the parental diet. Thus 4 experimental groups were used. The groups included progeny (fed control and rachitogenic diets) from control and vitamin C-treated parents.

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In the second study progeny from parents fed the control breeding diet or this ration supplemented with 20 mg of ascorbic acid per pound of diet were used. At one day of age the chicks were separated on a basis of parental diet and supplied with a rachitogenic diet similar to that previously described with the exception that the steamed bone meal was not removed. This procedure was followed because the rachitogenic diet in the first study was deficient in calcium, phosphorus and vitamin D_3. Thus, it was possible to observe the effect of vitamin D_3 deficiency in the dietary absence and presence of these two minerals. In each experiment the chicks were brooded in electrically heated batteries under management conditions previously described (Thornton et al., '59).

When the chicks were 16 days of age, they were injected with a 0.5-ml aqueous solution containing approximately 10 μc of Ca^{45} plus 15 mg of Evans Blue Dye. No carrier calcium was added to this dose. The solution was injected directly into the lumen of the crop after this organ was exposed by making a small skin incision. After injection the birds were returned to the battery where feed and water were available. The birds were killed at specific time intervals following injection of the aqueous solution. These intervals included: 30, 60, 120, 240, 480 and 1440 minutes in the first study; the 30-minute group was eliminated in the second experiment. Four individuals of each sex were used per treatment at each time interval. Immediately after sacrifice the individual body weight was determined and the "upper bowel" (esophagus, crop, proventriculus and ventriculus) and right tibia were removed for Ca^{45} analyses.

Prior to tibia ashing, the tibia epiphyseal-cartilaginous plate width was determined. The tibia and "upper bowel" were then ashed separately at 700°C. The tibia ash was next weighed and then taken up in a 5% nitric acid solution, transferred quantitatively to a volumetric flask and made to 100-ml volume. The "upper bowel" ash was treated similarly except no ash weight determinations were made. A 4-ml aliquot was taken and placed on a stainless steel planchet and evaporated to dryness at room temperature.

The Ca^{45} activity was determined in a gas flow, windowless-type scaler. Self-absorption as well as physical decay calculations were performed on each sample. The amount of Ca^{45} recovered from the tibia was corrected by calculating the tibia counts/minute per counts/minute of Ca^{45} passed from nonabsorptive to the absorp-

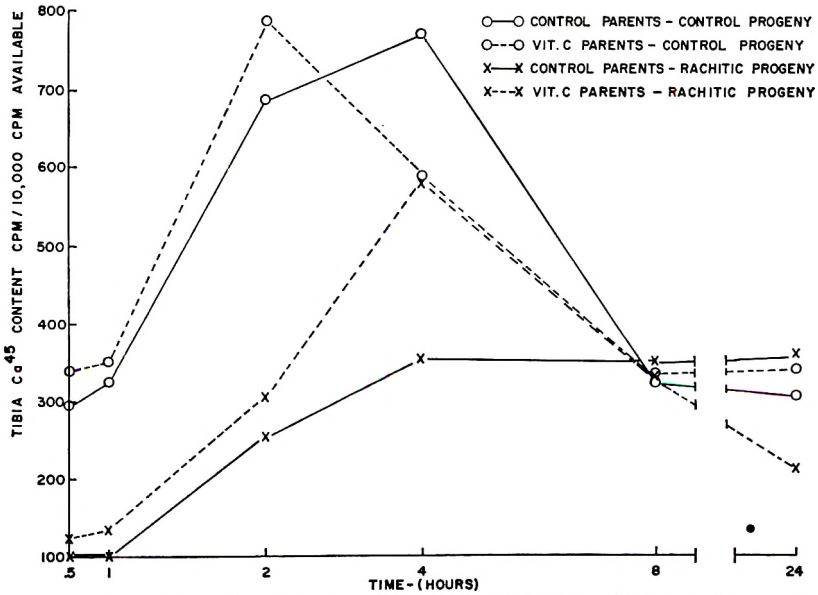

Fig. 1 The tibia Ca^{45} content as affected by intestinal transit.

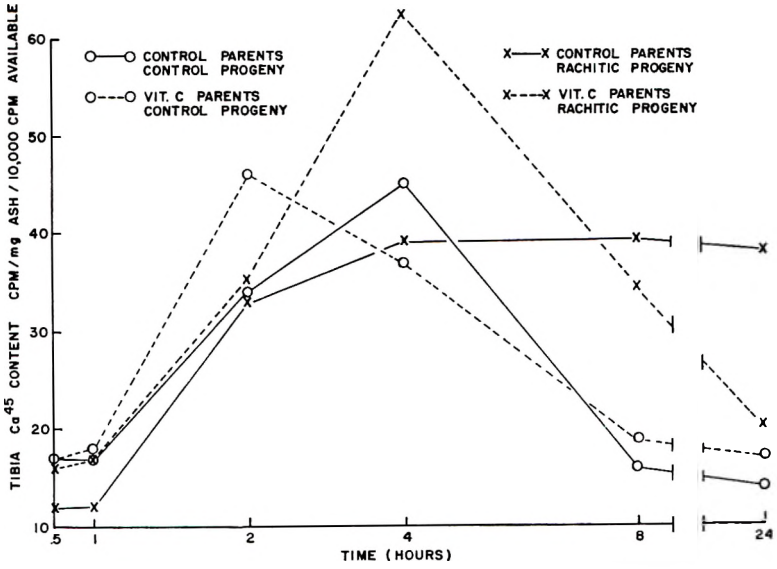

Fig. 2 The tibia Ca^{45} content as influenced by intestinal transit and skeletal ash weight.

tive portion of the bowel (fig. 1). This correction was made in view of the previous observation (Thornton et al., '59) that these factors were highly correlated. Data tabulated in this manner are presented graphically (fig. 1).

A second correction factor for bone ash weight was also introduced (fig. 2). This calculation was made to eliminate differences in bone ash weight and thus size of bone. The value represents the degree of concentration of Ca^{45} in the bone rather than total Ca^{45}. Such a value appears to be more indicative of the deposition potential of a bone for a given time without regard to previous treatment. The rachitic chicks had been limited by the dietary treatments imposed; thus, it seemed worthwhile to determine the amount of Ca^{45} which was deposited per unit of bone available for deposition.

RESULTS AND DISCUSSION

The bone ash (table 1) was reduced markedly in those chicks given the rachitogenic diet, as expected. Of interest are the values for the rachitic chicks in the two experiments, however. A high degree of similarity persisted despite the deficiency of calcium and phosphorus in the first experiment as compared with experiment two. These results suggested that the amount of bone salt formed was dependent on the presence of vitamin D_3 in the diet and that the inclusion of calcium and phosphorus was not involved. Whether this failure to form bone salts in each case was due to a reduced intestinal absorption of these minerals or to a direct effect of vitamin D_3 on bone metabolism is a point of conjecture. Parental ascorbic acid apparently had no effect on bone ash weight with the experimental variables imposed in these experiments.

Body weight was noticeably reduced by the absence of vitamin D_3 and the steamed bone meal in the first study. It was evident that the parental vitamin C had no influence on this factor in either control or rachitic progeny in this trial. A significant difference was observed, however, in the second study (table 1) between the two rachitic groups. In view of the reversal in trend for body weight between the two experiments, such data are questionable as to meaningfulness.

The presence of vitamin C in the parental diet appeared to influence the epiphyseal plate width measurements in both experiments (table 2). This was particularly apparent in the control male chicks from parents given vitamin C and the rachitic female chicks from the same parentage. In fact, all progeny of parents

TABLE 1

Effect of the rachitogenic diet on tibia ash and body weight

Dietary treatment	Tibia ash weight		16-Day body weight	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	mg/100 gm body wt.		*gm*	*gm*
Parental, control Progeny, control	128		157 ± 3.3[1]	
Parental, ascorbic acid[2] Progeny, control	128		154 ± 3.1	
Parental, control Progeny, rachitogenic	84	86	109 ± 2.8	132 ± 2.1
Parental, ascorbic acid Progeny, rachitogenic	85	85	113 ± 3.0	125 ± 2.6[3]

[1] Mean ± standard error of the mean.
[2] Ascorbic acid was supplemented to the parental diet at a level of 44 and 88 mg per kg in the first study and 44 mg in the second.
[3] Significantly different from the rachitic progeny from control parents to the 5% level of probability.

TABLE 2

Tibia epiphyseal plate width as influenced by the parental and progeny diets

Dietary treatment	Epiphyseal plate width			
	Males		Females	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	mm	*mm*	*mm*	*mm*
Parental, control Progeny, control	1.1 ± 0.03[1]		1.1 ± 0.13	
Parental, ascorbic acid[2] Progeny, control	1.8 ± 0.21[4]		1.2 ± 0.13	
Parental, control Progeny, rachitogenic	3.0 ± 0.25	1.3 ± 0.07	2.8 ± 0.19	1.2 ± 0.11
Parental, ascorbic acid Progeny, rachitogenic	3.3 ± 0.22	1.7 ± 0.11[3]	3.3 ± 0.16[3]	1.4 ± 0.07

[1,2] See table 1, footnotes 1 and 2.
[3] Significantly different from rachitic chicks from control parents to the 5% level of probability.
[4] Significantly different from the control progeny from control parents to the 1% level of probability.

receiving supplemental vitamin C showed a higher value for this factor than the corresponding group from control parents.

In normal growth of long bones a continuous multiplication and piling up of cartilage cells occurs, which results in the formation of the epiphyseal plate (Follis, '60). A continual deposition of lime salts in the matrix substance in the interstices of these cells also occurs with osteoid tissue being deposited on the lime salt matrix resulting in formation of bone by deposition of inorganic calcium and phosphorus. Thus it appears possible in this case that either the cartilage cells were deposited at a faster rate or that the conversion of cartilage cells to bone cells was inhibited. Since no histological studies were made, no definite conclusions can be drawn.

Of interest was the rather large standard error exhibited by the control males from parents fed the vitamin C, indicating that all individuals did not react similarly. Individual observations showed that 12 birds from this group had a value of 1.0 mm which was comparable to the control birds from control parents. The remaining 12 had an epiphyseal plate width value of 2.6.

Therefore, it appears that the response was very evident in some animals, but nonexistent in others. Since two levels of ascorbic acid supplementation were used in the parental diet, it is possible that the varying response can be attributed to this variation. Unfortunately, no attempt was made to segregate the progeny of parents fed different levels of ascorbic acid; thus this variable could not be measured in this instance.

The increased plate width of rachitic chicks from parents fed the vitamin C compared with rachitic chicks from control parents substantiates earlier work at this laboratory (Thornton et al., '59). In view of the increased epiphyseal plate widths observed in both control and rachitic chicks from parents fed vitamin C in this experiment, as well as the earlier observation, it seems possible that parental dietary ascorbic acid may have had some function in either the production of cartilage cells or their conversion to bone tissue.

Rate of movement of the Ca^{45} from the nonabsorptive portion of the digestive tract to the absorptive area is shown in table 3. There did not appear to be any differences between the two groups of chicks fed the control diet. Similarly the two groups fed the rachitogenic diet showed no differences. It did appear, however, that the rachitic chicks had a slower rate of transit than the control groups. This effect was in evidence through the first 4 hours following injection of the isotope.

The tibia Ca^{45} values for the 4 experimental groups are shown in figure 1. In this case the values are based on the total Ca^{45} recovered from the tibia and corrected for the amount of the isotope passing from the nonabsorptive to the absorptive area of the digestive tract.

The two groups given the control chick diets showed highly similar results (fig. 1). The group from the parents given vitamin C reached a maximal value earlier than the chicks from control parents. However, the total turnover of Ca^{45} for the control groups does not appear greatly different despite the evidence that the chicks from parents fed vitamin C first showed this effect.

Data for the rachitic chicks, the progeny of parents fed vitamin C (fig. 1), resulted in a curve very similar in shape to that for the two control groups to 8 hours postinjection. During the first 4 hours the values for this group were not of the magnitude shown by the control groups, however. These results disagree with those of Thornton et al. ('59), in which it was shown that rachitic and control birds from parents given the ascorbic acid deposited similar amounts of Ca^{45} in the tibia to one hour postinjection. Certain changes in the experimental procedure between these studies, such as age difference and dietary levels of calcium and phosphorus in the rachitogenic diets, could account for such differences, although this conclusion cannot be made without additional evidence. Between 8 and 24 hours postinjection the rachitic chicks from parents fed vitamin C showed a further loss of Ca^{45} from the bone. Since the two control groups exhibited practically no turnover of Ca^{45} during this period, it would seem that resorptive processes were more dominant in the rachitic group and may partially account

TABLE 3

Intestinal transit of calcium^{45}

Group[1]	Dietary treatment			
	Parental, control Progeny, control	Parental, ascorbic acid Progeny, control	Parental, control Progeny, rachitogenic	Parental, ascorbic acid Progeny, rachitogenic
	percentage of Ca^{45} leaving upper tract			
30-Minute	35.4	50.9	33.3	28.9
60-Minute	91.1	82.6	64.0	65.0
2-Hour	94.8	94.1	61.2	69.0
4-Hour	95.9	95.1	84.3	79.9
8-Hour	99.0	98.5	97.0	97.4
24-Hour	100.0	100.0	100.0	100.0

[1] Refers to time of sacrifice following injection of the isotope.

TABLE 4

Influence of parental ascorbic acid on skeletal turnover of Ca^{45} by rachitic progeny

Group	Control parents		Ascorbic acid-fed parents	
	Exp. 1	Exp. 2[1]	Exp. 1	Exp. 2[2]
hours				
1	12[3] ± 1.1	20 ± 2.3	17 ± 4.6	34 ± 2.1
2	33 ± 6.1	33 ± 7.5	35 ± 3.5	44 ± 9.7
4	39 ± 4.7	73 ± 4.7	62 ± 3.1	88 ± 7.6
8	39 ± 2.4	75 ± 6.5	34 ± 3.8	57 ± 5.7
24	38 ± 3.5	44 ± 4.7	20 ± 2.4	40 ± 4.5

[1] r = 0.784, significant at 5% level of probability.
[2] r = 0.963, significant at 1% level of probability.
[3] Count/min./mg of bone ash/10,000 count/min. of Ca^{45} passed to absorptive area of the digestive tract.

for the rachitic symptoms and low bone ash values exhibited by these birds.

Data for the rachitic chicks from control parents resulted in a curve (fig. 1) which was widely different from the three curves previously discussed. For example, the rate of Ca^{45} uptake was much slower, the maximal level reached was lower and there was no evidence that bone salt turnover was present as shown by the highly similar values between 4 and 24 hours. It was possible that resorption of Ca^{45} was taking place in this group between 4 and 8 hours since the group showed a retarded rate of intestinal transit (table 3). In such a case Ca^{45} deposition may have been equal to Ca^{45} resorption, yet the rachitic group from parents fed vitamin C showed a turnover effect despite an intestinal transit rate which was comparable to the rachitic birds from control parents. In view of these differences, it appeared that the presence of vitamin C in the parents' diet had a marked effect on the calcium metabolism in the chicks fed the rachitogenic diets.

A further correction for bone ash weight was made to eliminate bone size differences. These data (fig. 2) resulted in curves which were highly similar in shape to those shown in figure 1. The relative values between rachitic and control birds were changed by this correction, however. The high maximal value for the rachitic birds from parents fed the ascorbic acid indicated that these chicks were very efficient in depositing Ca^{45} in the skeleton. Since the total amount of Ca^{45} deposited in the tibia by this group was considerably less than that by the two control groups (fig. 1) it would seem that the limiting factor may have been deposition area in the rachitic birds.

In view of the marked difference in rate of biological turnover of Ca^{45} between the two rachitogenic groups in the first study, a second experiment was initiated to observe this factor. Although there were differences in the tibia Ca^{45} content between the two experiments, the results for the two studies were correlated (table 4). This was particularly noticeable in those chicks from parents given the vitamin C where the relationship was significant to a high degree of probability, whereas chicks from control progeny exhibited a value significant to the 5% level. The similarity in results between the two experiments suggests that the presence of vitamin C in the diet has an influence on both the amount of Ca^{45} deposited in the skeleton and the resorption of this mineral. Because the chicken can synthesize vitamin C, it is difficult to assume that such differences are a direct result of this vitamin. This is particularly so in this case since this effect was necessarily mediated through the egg.

Despite the ascorbic acid-synthesizing faculty of the avian species, Thornton ('59) showed that progeny of parents given supplementary ascorbic acid had a bone ash weight greater than that of the controls in a highly significant manner at one day of age. Other data[4] have shown that progeny from parents given vitamin C consistently have slightly greater values for calcium, phosphorus and hydroxyproline in the skeleton when hatched. Thus it seems probable that the developing embryo from parents fed the vitamin C was able to in-

[4] Thornton, P. A. 1961 Unpublished data.

corporate more of the egg's products into the skeleton during growth than comparable organisms from control parents.

In retrospect, it was evident that Ca^{45} utilization was widely different between the rachitic chicks from control parents and those given the vitamin C. This was reflected in both the amount of isotope deposited in the skeleton and in the biological turnover. Despite these differences, a high degree of similarity was noted in the skeletal ash values for the two groups. Since each was noticeably and equally rachitic, it seems probable that rickets developed from different causes in the two cases.

Inasmuch as the rachitic progeny from parents fed vitamin C were able to absorb more Ca^{45} than rachitic chicks from control parents, it seemed probable that the continued loss of Ca^{45} from the skeleton between 8 and 24 hours (fig. 1) for the former group was the basis for the rachitic development. From this it may be explained that these chicks developed rickets due to the predominating influence of the catabolic phase in bone salt metabolism. In the second case, the rachitic chicks from control progeny were unable to deposit Ca^{45} in the skeleton in a comparable manner; and furthermore, there was nearly a total lack of bone salt turnover between 4 and 24 hours, suggesting that the catabolic as well as the anabolic phase of bone metabolism was inhibited.

If these suppositions are correct, and the data from two separate experiments substantiate such views, then it appears probable that vitamin D was involved in the normal metabolism of the skeleton. In this respect it seemed possible that vitamin D acted in some manner to regulate the synthesis and resorption of bone salts.

SUMMARY

The influence of ascorbic acid in the parental diet on calcium utilization and bone development was studied using rachitic and control progeny.

The parental vitamin C appeared to influence epiphyseal plate width in control and rachitic progeny, with a consistent increase observed in progeny from parents fed the ascorbic acid. Whether this effect was the result of increased cartilage cell development or an inhibition in the conversion of cartilage to bone tissue is a point of conjecture.

Parental vitamin C appeared to influence calcium skeletal retention in the rachitic progeny. In this respect it appeared that the presence of this vitamin was associated with an increased amount of Ca^{45} deposition by the bone.

Between 4 and 8 hours postinjection, the two control groups and the rachitic group from parents fed vitamin C exhibited an active state of skeletal Ca^{45} loss. This effect subsided in the control groups after 8 hours but continued in the rachitic chicks to 24 hours. The rachitic chicks from control parents had a lower maximal Ca^{45} level in the skeleton and showed no evidence of loss of this isotope from this tissue to 24 hours postinjection. Despite these apparent differences between the two rachitic groups for Ca^{45} utilization by the skeleton, there were no differences in bone ash weight for the two groups. A possible explanation for this discrepancy is given in the discussion.

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Effect of Dietary Oleate and Linoleate on the Distribution of Fatty Acids in Mouse Triglycerides[1]

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Studies on the kinetics of the disappearance of linoleic acid from the depot fat of mice fed a fat-free diet revealed two rates of linoleic acid decline (Tove and Smith, '59). In addition, two patterns of deposition of linoleic acid were observed when safflower oil was fed (Tove and Smith, '60). It seemed possible that these observations might be associated with differences in the position of linoleic acid in the triglyceride molecule. The finding that the majority of the linoleic acid was esterified at the β position of the triglyceride (Savary et al., '57; Mattson and Lutton, '58) gave further support to this notion. The results of an investigation of the behavior of α- and β-esterified linoleic acid and oleic acid are reported in this communication.

EXPERIMENTAL

Animal procedure. The study on the kinetics of disappearance of linoleic acid from the depot fat of mice was conducted essentially as previously described (Tove and Smith, '59). The basal diet was modified in that the egg albumen and starch components were replaced by casein and sucrose. Safflower oil[2] at a level of 15% was fed to weanling male mice for three weeks to produce an elevated level of linoleic acid in the depot fat. After replacing the safflower oil diet with the fat-free diet, two animals were killed at three-day intervals and the depot fat was extracted (Tove and Smith, '59). In addition, two experiments were conducted in which a source of oleic acid was added to the casein-sucrose basal diet at levels of 1, 5, 10, 20 and 30%. In the first experiment triolein[3] was used and the depot fat was extracted from the carcass following removal of the head, skin and gastrointestinal tract. In the second experiment glyceryl mono-oleate[4] was fed and only the inguinal subcutaneous fat pads were extracted. This extraction was accomplished by grinding the fat pads with 10 ml of alcohol-ether (3:1) in a tissue homogenizer, bringing the homogenate to a boil and removing the protein by filtration.

The lipolysis procedure used was that described by Mattson and Beck ('55) except that tris buffer was used instead of ammonium chloride-ammonium hydroxide buffer. The hydrolyzed fatty acids were removed from the washed ether extract of the lipolysis medium by adsorption on Amberlite IRA-400 (Cason et al., '50). With samples from a previous linoleic acid experiment in which graded levels of safflower oil had been fed (Tove and Smith, '60) and from the oleic acid experiment in which the inguinal subcutaneous fat pads were used, the monoglycerides, diglycerides and triglycerides were separated by chromatography on silica gel (Quinlan and Weiser, '58). With samples from the linoleic acid experiment on kinetics of decline and from the oleic acid experiment in which the whole carcass

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[2] Kindly supplied by the Pacific Vegetable Oil Corp., San Francisco.
[3] Kindly supplied by Emery Industries Inc., Cincinnati.
[4] Kindly supplied by Distillation Products Industries, Rochester, New York. Myverol, distilled glycerol mono-oleate, Type 18–71, prepared from USP oleic acid.

was extracted, the glycerides were separated by Florisil chromatography (Carroll, '61). The fatty acid composition of triglycerides and monoglycerides was determined by gas chromatography of the methyl esters on a 4-foot column of Celite containing 30% succinate-ethylene glycol polyester (Tove and Smith, '60). The methyl esters of the fatty acids were prepared by transesterification of the glycerides. The glycerides (0.2 to 0.5 gm) were dissolved in a mixture of 2.5 ml of benzene, 20 ml of dry methanol containing 5% of hydrogen chloride, and 1 ml of 2,2-dimethoxypropane. The lipid solution was allowed to stand at room temperature overnight, and the methyl esters were extracted with hexane. The hexane extract was washed with water and simultaneously dried and neutralized by treatment with a mixture of anhydrous sodium sulfate and anhydrous sodium carbonate (4:1). The fatty acid composition of the monoglyceride fraction represents that of the β position (Mattson and Beck, '56). The fatty acid composition of the α positions was computed by the difference between the composition of the triglyceride and that of the β position.

RESULTS

Distribution of linoleic acid. In a previous experiment with mice (Tove and Smith, '60) percentage of linoleic acid in depot fat plotted against level of safflower oil fed resulted in a biphasic curve. Samples of fat saved from this experiment were pooled and the fatty acid composition of the α and β positions determined. The amount of linoleic acid in the intact triglyceride, the β position and the sum of the two α positions are shown in figure 1. Each point on the graph represents the average of two animals. As the dietary linoleate increased, there was a slight initial increase in β-esterified linoleic acid, after which the level remained relatively constant. The greatest part of the dietary linoleate, however, was deposited in the α positions in a manner similar to that observed with the intact triglyceride. At low dietary levels of safflower oil more linoleic acid was found in the β position than the average for either one of the α positions. Such a distribution is normally observed

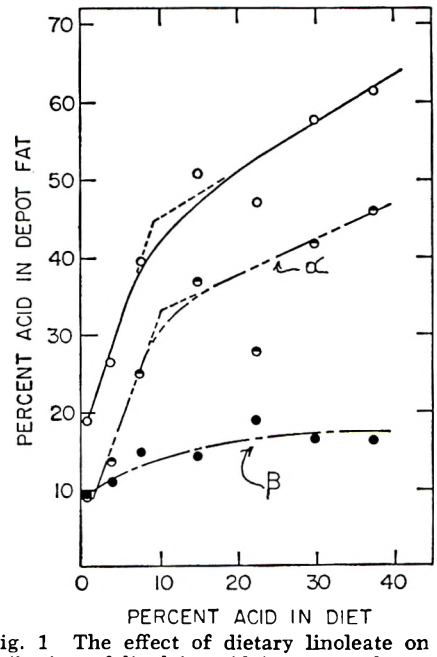

Fig. 1 The effect of dietary linoleate on the distribution of linoleic acid in mouse depot fat: ○, intact triglycerides; ◒, α positions; ●, β position.

with most triglycerides (Savary et al., '57; Mattson and Lutton, '58; McCarthy et al., '60). With high levels of safflower oil, however, the average linoleic acid esterified in a single α position exceeded that esterified in the β position.

The change in the level of linoleic acid in the α and β positions of the depot fat of mice following the removal of safflower oil from the diet is shown in figure 2. The decline of linoleic acid from the α position closely resembled that of the intact triglyceride (Tove and Smith, '59) and was initially faster than the decline of linoleic acid esterified at the β position. The average level of linoleic acid in a single α position exceeded that of the β position only at zero time, presumably before the effect of removal of dietary fat was manifested. Thereafter the linoleic acid content of the β position exceeded the level in an α position.

A biphasic analysis (Tove and Smith, '59) of the data shown in figure 2 as well as that of the intact triglycerides resulted in the first order rate constants given in table 1. It is apparent from these data that the behavior of the linoleic acid esterified

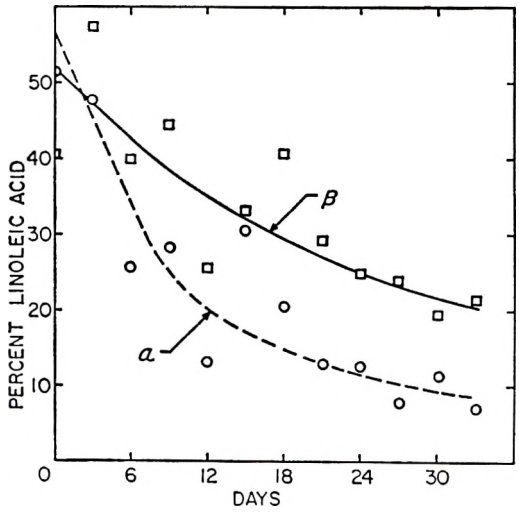

Fig. 2 Decline of linoleic acid from the α and β positions of the triglycerides of mouse depot fat: ○, average for an α position; □, β position.

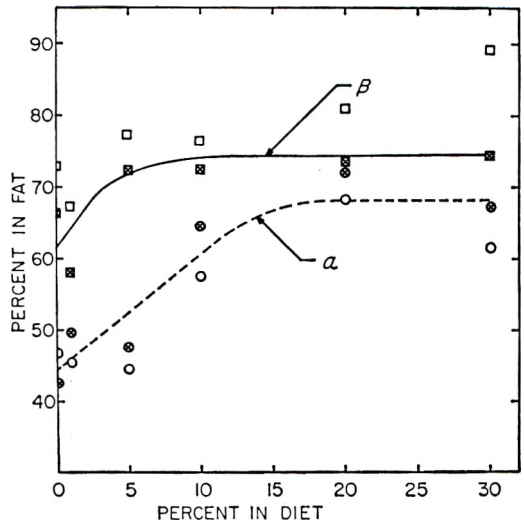

Fig. 3 Effect of dietary oleate on the distribution of oleic acid in mouse depot fat: ○, α positions of inguinal subcutaneous fat triglycerides, glyceryl mono-oleate fed; □, β position of inguinal subcutaneous fat triglycerides; ⊗, α positions total depot fat triglycerides, triolein fed; ⊠, β position total depot fat triglycerides.

in the β position was different from that in the α positions. Upon removal of the dietary source of linoleic acid, the rate of decline of linoleate from the α positions was similar to that for the intact triglycerides (Tove and Smith, '59). Furthermore, the rate of decline was faster for the first 20 days than for the latter part of the experiment, whereas the linoleate esterified in the β position moved out at a constant rate during both phases of the decline period. The slow rate of decline of the α-esterified linoleic acid was equal to that from the β position.

Distribution of oleic acid. The positional distribution of oleic acid in the carcass triglycerides of mice fed triolein (fig. 3) resembled the observations of the linoleic acid experiment in several respects. As in the case of linoleic acid the majority of the increase in oleic acid in depot fat observed was in the α position, reaching a maximum of 70% of the fatty acid in this position. The shape of this curve also closely resembles that observed with the intact triglyceride (Tove and Smith, '60). As with linoleate, the β position was found to be relatively inert compared with the α, and except for the initial rise the level in the β position remained constant. The major difference between the oleate and linoleate results was that the level of β-esterified oleate always exceeded that of an α position irrespective of the amount in the diet.

TABLE 1

First order rate constants of the decline of linoleic acid from α and β positions of the depot fat triglycerides

	Time after safflower oil ingestion	
	< 20 Days	> 20 Days
Intact triglyceride	−0.042	−0.024
α Position	−0.057	−0.019
β Position	−0.019	−0.020

The distribution in the triglycerides of subcutaneous inguinal fat pads from mice fed glyceryl mono-oleate was essentially the same as that of the triglycerides of the whole carcass from mice fed triolein (fig. 3). This similarity suggests that essentially the same pattern of deposition probably exists among the various adipose tissue sites and confirms the observation (Tove and Smith, '60) that the dietary form of oleate does not influence its pattern of deposition.

Fatty acid distribution in mouse depot fat. The percentages of each of the 6 principal fatty acids found in the β position of triglycerides of mice are given in

TABLE 2
Distribution of fatty acids in mouse depot fat

Experiment	Fatty acids					
	14:0	16:0	16:1	18:0	18:1	18:2
	% in β position[1]					
"Linoleic-fed"	19.4	17.7	27.8	22.0	40.0	52.3
"Linoleic decline"	29.0	23.6	39.6	18.8	34.9	42.0
"Oleic total depot fat"	31.0	21.9	31.2	71.5	38.1	53.6
"Oleic fat pads"	26.7	20.4	32.2	21.4	39.2	49.3
Average	26.5	20.9	32.7	20.7[2]	38.0	49.3

[1] Grams of specific fatty acids in the β position per 100 gm of that fatty acid in the total triglycerides (including both positions).
[2] Value for 18:0 acid obtained in "oleic total depot fat" experiment omitted from average.

table 2. The averages given in table 2 were compiled from 4 experiments. Only groups receiving the low levels of dietary fat were included since they were considered more nearly normal. These were: mice fed either zero or 1% of fat in the linoleate feeding experiment and in the two oleate experiments, and mice receiving the fat-free diet for 27 days or longer in the linoleate decline experiment. The distribution of fatty acids of mouse depot fat was similar to that reported for other species (Savary et al., '57; Mattson and Lutton, '58; McCarthy et al., '60). The saturated acids were predominantly esterified at the α positions and the unsaturated acids, at the β position. A value of 33% would indicate equal or random distribution. Only palmitoleic acid, with an average of 32.7% esterified at the β position, can be considered as randomly distributed in the glyceride structure. An unusual distribution of stearic acid was found in the fat of mice from the oleate experiment in which triolein was fed. Unfortunately, there is no readily apparent explanation for this enigma.

DISCUSSION

In recent years it has been shown that the triglycerides of several animal and plant species do not contain a uniform distribution of fatty acids among the three positions of the triglyceride molecule (Savary et al., '57; Mattson and Lutton, '58; McCarthy et al., '60). The results of the studies reported herein confirm these observations with mouse triglycerides. As with most animal fats, the unsaturated acids were predominantly esterified at the β position of the triglycerides and the saturated acids were predominantly esterified at the α positions. Palmitoleic acid was an exception as it was found to be uniformly distributed throughout the glyceride molecule. An unexpected result was the finding that the normal positional distribution of linoleic acid was reversed when high levels of safflower oil were fed. Although a similar reversal was not observed with oleic acid, it is nevertheless apparent that the specific distribution of a fatty acid in a triglyceride can be altered by diet.

The nonrandom distribution of fatty acids in animal triglycerides and the fact that a biphasic response was observed in both linoleic deposition (Tove and Smith, '60) and linoleic decline (Tove and Smith, '59) suggested that the different positions of the glyceride might be metabolized at different rates. The results of the studies in which graded levels of linoleate and oleate were fed demonstrate that the α and β positions differ markedly with respect to the deposition of these fatty acids. With both dietary treatments, the greatest change in fatty acid distribution was observed in the α positions, whereas, except for an initial increase when the amount of fat in the diet was low, the level in the β position remained relatively constant. Alternately, although both the fast and slow rates of linoleate disappearance observed with the intact triglycerides also were exhibited by the α-esterified linoleate, the linoleate at the β position exhibited only the slow rate of decline.

From these results, the likelihood that the deposition and depletion of linoleic acid, as well as the deposition of oleic acid, reflect differences in rate of metabolism seems reasonable. Presumably, the fatty

acids in the β position are metabolized at a slower rate than those in the α positions.

There are two principal routes by which a dietary fatty acid might be incorporated into the triglycerides of depot fat. The most obvious pathway would be synthesis of the triglyceride from glycerophosphate and the fatty acyl-coenzyme-A esters (Weiss et al., '60). The second route would be by way of a lipase-catalyzed exchange of a fatty acid with a preformed glyceride. In this connection it has been demonstrated that pancreatic lipase (Borgström, '54) and lipoprotein lipase (Borgström and Carlson, '57) catalyzed the exchange of oleic acid with triolein and that the α positions were the sites of interchange. Furthermore, it has been shown (Shapiro et al., '56) that when adipose tissue was incubated with radioactive stearate, there was an exchange between the fatty acid of the medium and the glycerides of the adipose tissue.

A lipase-catalyzed exchange reaction involving only the α positions could account for the findings of greater reactivity of the α positions compared to the β position. Incorporation into the β position would then reflect *de novo* synthesis. Thus it is possible to account for the findings reported herein if one assumes that both pathways are in operation. With such an assumption the α positions could be expected to exhibit two rates of metabolism whereas the β position would show only one, and this would be the same as one of the α rates. The kinetics of the decline of linoleic acid are in accord with such a supposition.

SUMMARY

Oleic acid and linoleic acid were predominantly esterified at the β position of normal mouse triglycerides, whereas myristic acid, palmitic acid and stearic acid were more apt to be found in an α position. Palmitoleic acid was noted to be uniformly distributed in the triglycerides.

Deposition in the α position accounted for the greatest part of the increase in depot fat level of oleic and linoleic acids that accompanied the feeding of these acids. With high dietary linoleate levels the normal linoleic acid distribution was reversed and the amount in a single α position exceeded that of the β position. When mice were fed a fat-free diet, two rates of disappearance were observed for linoleic acid esterified at the α positions whereas only the slower rate was noted for β-esterified linoleic acid. These results support the thesis that the glycerides of depot fat are not formed in a uniform or random manner.

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ANNOUNCEMENTS

AMERICAN INSTITUTE OF NUTRITION

26th Annual Meeting *Atlantic City, N. J.*

April 14 (9 AM) to April 19 (12 N), 1962

Abstracts of papers to be presented at the meeting must be in the hands of the Secretary, Arnold E. Schaefer, Building 16-A, National Institutes of Health, Bethesda 14, Maryland by December 30, 1961.

APPLICATIONS FOR AIN MEMBERSHIP

Forms for AIN membership application, along with the necessary requirements and instructions, may be obtained by writing to the Secretary.

AMERICAN SOCIETY FOR CLINICAL NUTRITION

Second Annual Meeting *Colton Manor Hotel*

Atlantic City, April 28 — 1 to 5 PM

Investigators are encouraged to submit abstracts for consideration by the program committee. These abstracts should be limited to 300 words or less and 7 copies submitted on or before February 1, 1962 to the Secretary, Robert E. Hodges, M.D., University Hospitals, Iowa City, Iowa.

NUTRITION SOCIETY OF CANADA

At the Fourth Annual Meeting of the Nutrition Society of Canada, held at the Ontario Agricultural College, Guelph, on May 30th, the following officers were elected: President, Dr. E. H. Bensley, Montreal; Vice President, Dr. R. H. Common, Macdonald College; Treasurer, Dr. J. A. Campbell, Ottawa; Secretary, Prof. E. V. Evans, Guelph; Councillors, Dr. J. M. Bell (1964), Saskatoon, Dr. L. P. Dugal (1963), Ottawa, and Dr. W. W. Hawkins (1962), Halifax. Past President is Professor J. Biely, Vancouver.

A feature of the annual dinner of the Society was the announcement of the winner of the second Borden Award of the Nutrition Society of Canada for research in nutrition. This year's winner, Dr. Donald Fraser of the Research Institute, Hospital for Sick Children, Toronto, Ontario, was cited for his work on vitamin D-refractory rickets in children and on various aspects of calcium and phosphorus metabolism.

The special speaker at the dinner was Dr. Ross A. Chapman, Assistant Director, Scientific Services, Food and Drug Directorate, Ottawa, whose topic was "Research in the Food and Drug Directorate." The one-day Society program included a session of short research papers and a symposium on "Current Problems in Nutrition." Registered attendance at the meeting was 88 members and visitors.