Effect of Type of Dietary Fat on Plasma and Liver Cholesterol Concentration in Female Chicks[1]

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Studies by a number of investigators have shown that dietary fat, protein and minerals can affect blood cholesterol levels in growing chicks. March and Biely ('59) reported that hydrogenated vegetable fat[2] butter, lard and chicken fat, but not herring oil, increased serum cholesterol levels in growing chicks. Leveille and Fisher ('58) observed that cockerels fed a 25% protein diet supplemented with 2% of cholesterol maintained essentially normal plasma cholesterol levels regardless of level or source of dietary fat. Dam et al. ('55) reported that a marked increase in liver cholesterol in chicks occurred when supplementary cholesterol was added to a diet containing 10% of peanut oil. Hegsted et al. ('60) showed that fats high in saturated fatty acids promote hypercholesterolemia in cholesterol-fed cockerels and this effect is counteracted by unsaturated fatty acids. Stamler et al. ('59) reported that hypercholesterolemia is similar in cholesterol-fed cockerels ingesting unsaturated or saturated fats; furthermore, supplementing the cholesterol containing diet with large amounts of unsaturated oils rich in essential fatty acids failed to suppress hypercholesterolemia. Wood and Biely ('60) noted that lingcod liver oil and halibut liver oil prevented the hypercholesterolemic effect of dietary cholesterol, whereas crude herring oil, corn oil and tallow increased cholesterol levels; and Piefer et al. ('60) observed that feeding tuna or menhaden oil was effective in lowering plasma cholesterol levels in hypercholesterolemic rats.

A study of factors affecting hepatic cholesterol synthesis by Curran ('54) led to the observation that low concentrations of vanadium salts exerted a marked inhibitory effect on hepatic cholesterol synthesis in rats. Curran and Costello ('56) reported that vanadium inhibited hepatic cholesterol synthesis in rabbits. Similarly, Mountain et al. ('56) noted that vanadium would reduce the liver tissue cholesterol content of rabbits fed 1% of cholesterol.

The studies reported here were conducted to determine whether the type of fat added to the diet of cholesterol-fed female chicks would result in plasma and liver cholesterol changes similar to those reported to occur in male chicks. The effect of vanadium on plasma and liver cholesterol was also studied.

EXPERIMENTAL

Day-old female chicks originating from a mating of New Hampshire males to Barred Plymouth Rock females were used in all trials. They were housed in electrically heated batteries with raised-wire floors. Feed and water were supplied ad libitum. The basal diet used in all trials is shown in table 1. The iodine absorption values (AOAC, '55) of the fat and oils used in these trials were menhaden oil (203), tuna oil (203), sardine oil (160), redfish oil (129), refined corn oil (125), winterized cottonseed oil (115), and lard (66).[3] Vanadium was supplied as $VOCl_2$. Ten chicks were used per treatment. All trials were terminated when the chicks were 4 weeks of age. At the end of the 4th week, the chicks were kept off feed for 18 hours prior to collection of blood for cholesterol determinations. Livers were removed immediately after the chicks were killed, and stored at $-20°C$ until analyzed for cho-

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[2] Crisco, Procter and Gamble, Cincinnati.

[3] The fish oils were acquired through the cooperation of Dr. David Miller, Maine Marine Products Inc., Portland, Maine.

lesterol content. Cholesterol was determined on duplicate samples of plasma or liver tissue of individual chicks by the method of Koval ('61). The data were analyzed by the method of Duncan ('55).

TABLE 1

Composition of basal diet

	%
Ground yellow corn	60.00
Soybean oil meal (44% protein)	35.50
Steamed bonemeal	3.00
Ground limestone	0.50
Salt (4% $MnSO_4$)[1]	0.50
Choline chloride supplement (25%)	0.15
DL-Methionine	0.20
Vitamin B_{12} supplement (6 mg/pound)	0.10
Stabilized vitamin A (10,000 USP/gm)	0.11
Stabilized vitamin E (20,000 IU/pound)	0.07
	mg/kg
3 Nitro-4 hydroxyphenylarsonic acid	20
Chlortetracycline	20
Stabilized vitamin D_3 (15,000 ICU/gm)	60
Potassium iodide	26
Ca pantothenate	2
Niacin	6
Riboflavin	4
Menadione	2

[1] Ninety-six parts NaCl and 4 parts $MnSO_4 \cdot H_2O$.

RESULTS AND DISCUSSION

Trial 1. The results of this trial are summarized in table 2. The addition of 0.5% of cholesterol to the basal diet resulted in a significant increase in cholesterol content of both plasma and liver. Addition of 3% of redfish oil to the cholesterol-supplemented diet had no effect on plasma cholesterol, but significantly increased liver cholesterol. No significant reduction in plasma or liver cholesterol occurred when 50 ppm of vanadium were added to the basal diet or the cholesterol-supplemented diet.

Feed consumption and growth rate were not affected by cholesterol supplementation. The addition of 50 ppm of vanadium to the diet, however, reduced feed consumption by approximately 25%, resulting in a significant reduction in growth rate.

Trial 2. The results of trial 2 are summarized in table 3. Feeding 50 ppm of vanadium with and without supplementary cholesterol gave plasma and liver cholesterol concentrations similar to those obtained in trial 1.

TABLE 2

Trial 1. Effect of cholesterol, vanadium, and redfish oil on growth, feed conversion, plasma and liver cholesterol content of 4-week-old chicks (10 chicks/dietary treatment)

Group no.	Dietary treatment	Av. 4-week body weight	Feed consumption Feed/gain	Plasma cholesterol	Liver cholesterol
		gm	*pound/pound*	*mg/100 ml*	*mg/gm (wet-weight)*
1	Control	332	1.80	137 ± 19[1]	3.9 ± 0.7[1]
2	Control plus 0.5% cholesterol	319	1.86	198 ± 56	6.9 ± 2.4
3	Control plus 50 ppm vanadium	248	2.00	127 ± 15	3.9 ± 0.7
4	Control plus 0.5% cholesterol and 50 ppm vanadium	240	2.06	154 ± 50	5.1 ± 1.8
5	Control plus 0.5% cholesterol and 3% redfish oil	330	1.86	200 ± 68	10.4 ± 5.6

Duncan's multiple range test[2]

Growth (5% level of significance)

Group no.	4	3	2	5	1
Mean	240	248	319	330	332

Plasma cholesterol (1% level of significance)

Group no.	3	1	4	2	5
Mean	127	137	154	198	200

Liver cholesterol (5% level of significance)

Group no.	3	1	4	2	5
Mean	3.9	3.9	5.1	6.9	10.4

[1] Standard deviation.

[2] Means underscored by same line are not significantly different at levels of probability indicated.

The addition of either corn oil or cottonseed oil to the cholesterol-supplemented diets significantly increased plasma cholesterol levels above the level observed when no oil was added. These data are in agreement with March and Biely ('59) that hypercholesterolemia can be induced in growing chickens by supplementing a 20% protein diet with corn oil and cholesterol. Jones et al. ('56) have reported that higher serum cholesterol levels were obtained when cottonseed oil instead of corn oil was fed to chicks receiving a cholesterol supplement. In this trial we observed a slight but not significant increase in plasma cholesterol when the feeding of cottonseed oil was compared with the feeding of corn oil to chicks receiving the cholesterol supplement. (In trial 3 no difference was observed in plasma cholesterol levels when either corn or cottonseed oil was fed.) No significant increase in plasma cholesterol resulted from adding fish oil to the cholesterol-supplemented diet. These observations are similar to those of Wood and Biely ('60) who reported that certain fish liver oils are effective in preventing hypercholesterolemia resulting from supplementing the diet with cholesterol. Stamler et al. ('59) also reported that hypercholesterolemia was less marked with fish oils than with cottonseed oil.

Feeding the relatively more saturated redfish oil (iodine absorption value 129) resulted in a significantly lower liver cholesterol content than when either tuna or menhaden oil (iodine absorption value 203) was added to the cholesterol-supplemented diet. There were no significant differences in the liver cholesterol content of the tuna, menhaden, corn oil or cottonseed oil fed groups.

As seen in trial 1 the addition of 50 ppm of vanadium to the diet with and without supplementary cholesterol resulted in a significant reduction in growth.

TABLE 3

Trial 2. Effect of dietary cholesterol, vanadium and oils on growth, feed conversion, plasma and liver cholesterol levels of 4-week-old chicks (10 chicks/dietary treatment)

Group no.	Dietary treatment	Av. 4th week weight	Feed conversion Feed/gain	Plasma cholesterol	Liver cholesterol
		gm	*pound/pound*	*mg/100 ml*	*mg/gm (wet-weight)*
1	Control	313	1.94	146 ± 10[1]	3.7 ± 0.9
2	Control plus 0.5% cholesterol	334	1.92	221 ± 39	8.6 ± 2.8
3	Control plus 50 ppm vanadium	232	2.11	132 ± 20	3.9 ± 0.2
4	As 2 plus 3.0% tuna oil	366	1.77	237 ± 63	24.4 ± 8.8
5	As 2 plus 3.0% redfish oil	355	1.78	183 ± 43	13.4 ± 5.0
6	As 2 plus 3.0% menhaden oil	347	1.85	211 ± 54	22.1 ± 6.8
7	As 2 plus 3.0% corn oil	367	1.80	369 ± 116	19.5 ± 6.8
8	As 2 plus 50 ppm vanadium	225	2.44	198 ± 83	5.4 ± 1.3
9	As 2 plus 3.0% cottonseed oil	362	1.81	390 ± 123	18.7 ± 9.2
10	Control	319	1.96	150 ± 17	3.9 ± 0.3

Duncan's multiple range test[2]

Growth (5% level of significance)

Group no.	8	3	1	10	2	6	5	9	4	7
Mean	225	232	313	319	334	347	355	362	366	367

Plasma cholesterol (5% level of significance)

Group no.	3	1	10	5	8	6	2	4	7	9
Mean	132	146	150	183	198	211	221	237	369	390

Liver cholesterol (1% level of significance)

Group no.	1	10	3	8	2	5	9	7	6	4
Mean	3.7	3.9	3.9	5.4	8.6	13.4	18.7	19.5	22.1	24.4

[1] Standard deviation.

[2] Means underscored by same line are not significantly different at levels of probability indicated.

Trial 3. The results of this trial are shown in table 4. The addition of lard or any of the oils to the basal diet did not increase plasma or liver cholesterol concentrations above the levels observed in the control groups of trials 1 and 2 receiving only the basal diet.

Although the plasma cholesterol levels of the cholesterol-fed groups receiving either corn oil or cottonseed oil were approximately 20% lower than when lard was fed, this difference was not statistically significant. In this trial, as in trial 2, the plasma cholesterol levels of the cholesterol-supplemented groups fed fish oils were approximately 50% lower than when vegetable oils were fed. This difference was significant at the 1% level of probability.

As observed in trial 2, the cholesterol-supplemented group receiving redfish oil had a significantly lower liver cholesterol content than observed for the groups fed either menhaden or tuna oil. There were no significant differences in liver cholesterol content of the groups receiving cholesterol supplements when fed either lard, corn oil, cottonseed oil, menhaden oil or tuna oil.

No significant differences in growth rate resulted from feeding either fish oil, lard, or vegetable oils.

These trials showed that both plasma and liver tissue cholesterol concentration can be altered by the type of fat in the diet of chicks receiving supplementary cholesterol. The variation of liver and plasma cholesterol within groups increased as the

TABLE 4

Trial 3. *Effect of type of fat and oil on growth, feed conversion, plasma and liver cholesterol levels of 4-week-old chicks (10 chicks/ dietary treatment)*

Group no.	Dietary treatment	Av. 4th week weight	Feed conversion Feed/gain	Plasma cholesterol	Liver cholesterol
		gm	*pound/ pound*	*mg/100 ml*	*mg/gm (wet-weight)*
1	3% Corn oil	348	1.86	141 ± 10[1]	3.4 ± 0.2[1]
2	3% Corn oil + 0.5% cholesterol	326	1.87	418 ± 162	28.9 ± 11.0
3	3% Lard	349	1.86	134 ± 16	3.6 ± 0.3
4	3% Lard + 0.5% cholesterol	356	1.86	511 ± 147	23.1 ± 10.9
5	3% Tuna oil	352	1.80	115 ± 14	3.2 ± 0.2
6	3% Tuna oil + 0.5% cholesterol	373	1.85	195 ± 57	27.2 ± 9.5
7	3% Redfish oil	328	1.85	126 ± 10	3.6 ± 0.2
8	3% Redfish oil + 0.5% cholesterol	339	1.83	243 ± 97	13.6 ± 6.3
9	3% Menhaden oil	368	1.77	109 ± 21	3.3 ± 0.3
10	3% Menhaden oil + 0.5% cholesterol	376	1.87	266 ± 134	23.9 ± 11.7
11	3% Sardine oil	336	1.87	130 ± 10	3.5 ± 0.3
12	3% Sardine oil + 0.5% cholesterol	349	1.81	230 ± 89	15.3 ± 6.9
13	3% Cottonseed oil	353	1.86	148 ± 13	3.3 ± 0.2
14	3% Cottonseed oil + 0.5% cholesterol	355	1.83	420 ± 163	21.3 ± 11.5

Duncan's multiple range test[2]

Growth (1% level of significance)

Group no.	2	7	11	8	1	3	12	5	13	14	4	9	6	10
Mean	326	328	336	339	348	349	349	352	353	355	356	368	373	376

Plasma cholesterol (1% level of significance)

Group no.	9	5	7	11	3	1	13	6	12	8	10	2	14	4
Mean	109	115	126	130	134	141	148	195	230	243	266	418	420	511

Liver cholesterol (1% level of significance)

Group no.	5	13	9	1	11	7	3	8	12	14	4	10	6	2
Mean	3.2	3.3	3.3	3.4	3.5	3.6	3.6	13.6	15.3	21.3	23.1	23.9	27.2	28.9

[1] Standard deviation.

[2] Means underscored by same line are not significantly different at level of probability indicated.

cholesterol levels increased. For example, in trial 3, the coefficient of variance for the plasma cholesterol of the groups fed fat was 10.6% as compared with 37.8% for the groups fed both fat and cholesterol.

In these trials there was no apparent relationship between the degree of unsaturation of the fat fed and the plasma cholesterol levels of the cholesterol-fed chicks. Although the iodine absorption values of the fish oils fed ranged from 129 to 203, the plasma cholesterol levels of the chicks fed fish oils plus cholesterol were similar. Furthermore, the plasma cholesterol levels of the cholesterol-fed chicks receiving fish oils were only slightly higher than when fish oils were omitted. Feeding corn oil with approximately the same iodine absorption value as redfish oil in conjunction with cholesterol resulted in plasma cholesterol levels that were twice the level observed when redfish oil and cholesterol were fed. Since corn and cottonseed oils are a good source of linoleic acid, it is apparent that linoleic acid had no beneficial effect in reducing plasma cholesterol levels of the hypercholesterolemic chicks. There was no direct relationship between plasma cholesterol level and liver tissue cholesterol concentration in the case of fish oils. Groups fed cholesterol plus menhaden or tuna oils had low plasma and high liver cholesterol concentrations, whereas groups receiving redfish oil had low cholesterol concentrations in both plasma and liver. It has been demonstrated by Alfin-Slater et al. ('54) and Grunbaum et al. ('57) that under certain dietary treatments the concentration of cholesterol in plasma and liver are independent.

The addition of vanadium to the cholesterol-supplemented diet resulted in a slight but not significant reduction in both plasma and liver cholesterol concentration. This reduction appeared to be due to reduced dietary intake of cholesterol resulting from decreased feed consumption, rather than a vanadium-induced inhibition of cholesterol synthesis.

SUMMARY

Female chicks were fed a basal diet supplemented with 0.5% of cholesterol and 3% of fat or oil. The addition of 3% of fat or oil to the diet without cholesterol supplementation did not increase plasma or liver cholesterol concentration. When 0.5% of cholesterol was added to the diet, the type of oil fed had a definite effect on plasma and liver cholesterol concentration. There was no significant difference in the plasma cholesterol level of groups receiving the cholesterol supplement and fed either lard or vegetable oil. These groups, fed either lard or vegetable oils, had significantly higher plasma cholesterol levels than any of the groups fed fish oil. Although adding fish oils to the diet of cholesterol-fed chicks resulted in lower plasma cholesterol levels than when other oils were fed, only the groups fed redfish oil had a significantly lower liver cholesterol concentration. No significant reduction in plasma or liver cholesterol resulted from adding vanadium to the diet. Both feed consumption and rate of growth were decreased by the addition of 50 ppm of vanadium to the diet.

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Intestinal Synthesis of Riboflavin in the Rat[1]

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The symbiotic relationship between the mammal and its intestinal flora has been demonstrated repeatedly. Fridericia et al. ('27) reported that certain rats that were fed raw potato starch as the source of carbohydrate could survive and grow in the absence of a dietary supply of B-vitamins. Further, Coates et al. ('46) and Ford et al. ('53) maintained growth in rats with a riboflavin-deficient diet when raw potato starch was utilized as the carbohydrate; but when cooked starch, which was more digestible, was used, it could not support growth. The inclusion of sulfa drugs in the diet also destroyed the effectiveness of the starch in promoting intestinal synthesis of riboflavin. De and Roy ('51) observed that excretion of riboflavin in the urine and feces increased when lactose or starch replaced sucrose in a diet devoid of riboflavin and decreased when sulfa drugs were included in the diet. More recently Morgan and Yudkin ('57) and Haenel et al. ('59) have shown that diets containing sorbitol or lactose in place of sucrose promote the synthesis of sufficient B-vitamins to maintain almost normal growth rates in rats.

These alterations in the activity of microorganisms in the gastrointestinal tract demonstrate clearly that riboflavin produced there is available to the animal and may, under special conditions, meet its requirement for both maintenance and growth. The contribution of the intestinal synthesis of riboflavin, when measured in terms of growth, is negligible when riboflavin-deficient diets containing sucrose are used. This affords an opportunity to measure the contribution of intestinal synthesis to the maintenance requirement since weanling rats fed a riboflavin-deficient diet maintain their weight for several weeks.

Information about the rate of intestinal synthesis of riboflavin by measurement of fecal excretion alone does not give an adequate measure of total synthesis, since as Schweigert et al. ('45) and others have pointed out, the amounts of riboflavin absorbed or destroyed cannot be differentiated.

In this paper we describe a procedure for estimating the rate of destruction of riboflavin in the riboflavin-deficient rat by the use of D-riboflavin-2-C^{14} (Haley and Lambooy, '54). By correcting the apparent rate of synthesis for the rate of destruction, we obtain a measure of the total rate of intestinal bacterial synthesis of riboflavin.

METHODS

Weanling, female Wistar rats were housed and maintained as described before and supplied with a diet (Lambooy and Aposhian, '60) supplemented with 9 μg of D-riboflavin-2-C^{14} per gm.[2] Upon reaching adulthood these rats were bred and the resultant litters were weaned at 21 days.

As the litters became available, one or more of each litter were sacrificed and prepared for analysis as "initial" animals. Their littermates, as experimental animals, were placed in individual glass and plastic metabolism cages with provision made for separation of urine and feces. The animals in metabolism cages received a riboflavin-deficient diet (Lambooy and Aposhian, '60) ad libitum and the quantity consumed was measured. Urine was collected in amber bottles and protected from bacterial contamination by toluene, and the feces were removed frequently and maintained dry, in the cold, until prepared for analysis. The initial group (zero time)

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[2] The specific activity of the D-riboflavin-2-C^{14} used was 3.4 μc/mg or 1.26 mc/mmole.

consisted of 14 animals of either sex, whereas the animals that were maintained in the metabolism cages for two, 4, 6 and 8 weeks consisted of 10, 10, 11, and 10 animals,[3] respectively, of either sex.

Analyses. For analysis of total tissue and C^{14}-riboflavin content, the animals were killed at the end of two-, 4-, 6- and 8-week periods with an overdose of ether and tissues prepared for analysis by the procedure of Lambooy and Aposhian ('60). Urine samples were diluted minimally by acidifying with the appropriate amount of concentrated HCl to give a concentration of 0.1 N and were then autoclaved. After adjustment to pH 4.5, the solutions were made up to the nearest readily measureable volume, filtered and stored under toluene for analysis. Feces were hydrolyzed using 50, 100, 150 and 200 ml of 0.1 N HCl for the two-, 4-, 6- and 8-week fecal collections, respectively. After adjustment to pH 4.5, the volume was diluted to twice the volume of the hydrolysate, filtered and stored under toluene for analysis. Two-hundred-gram portions of each batch of the riboflavin-deficient diet were hydrolyzed in 150 ml of 0.1 N HCl and made up to two liters after adjusting to pH 4.5. An aliquot of this solution was concentrated by lyophilization in order to obtain a solution of appropriate concentration for analysis.

The amount of riboflavin in aliquots of samples of tissue, urine, feces and diet hydrolysates was determined by the usual microbiological procedure using *Lactobacillus casei* as the test organism (Association of Vitamin Chemists, '51).

Assay for radioactivity. All measurements were made with a gas-flow counter using the proportional region. The procedure of Berson and Yalow ('60) was modified slightly and used to correct for self-absorption. One-milliliter aliquots of the tissue, urine or feces hydrolysates were placed in stainless steel planchets, dried in an oven at 70°C and counted for a time sufficient to give 2,000 to 3,000 total counts and a counting rate, "A." To these same planchets were added one milliliter of a pure standard C^{14}-riboflavin solution with a concentration of 0.515 μg per ml. The concentration of the standard was checked by a microbiological assay which made it possible to correlate corrected counting rate with microbiological activity. The counting rate obtained with the standard C^{14}-riboflavin solution plus the sample gave a counting rate, "B." Then $\frac{A}{B-A} \times S \times D$, where S is the amount of C^{14}-riboflavin added in micrograms and D is the dilution factor, gives the amount of labeled riboflavin in micrograms that would give the observed counting rate, "A," corrected for self-absorption. All samples were assayed in quadruplicate and the Chauvenet criterion (Calvin et al., '49) was used to reject a determination when the deviation from the mean of the 4 determinations by a suspected result was greater than 1.54 times the standard error of the counting rate (determined from the square root of the total number of counts).

Samples of tissue hydrolysate prepared from nonlabeled tissues were made to contain varying quantities of C^{14}-riboflavin and assayed. A satisfactory correlation was obtained between the expected counting rate and the observed counting rate.

The depletion of C^{14}-riboflavin from the tissues. The quantity of C^{14}-riboflavin observed in the tissues of each animal was added to the quantity of riboflavin in the urine of the same animal to correct for loss of riboflavin in the urine. Means of the values, R, thus obtained for the two-, 4-, 6- and 8-week groups were used to give a measure of the residual C^{14}-riboflavin that differs from the initial quantity of C^{14}-riboflavin, I, by the amount destroyed.

The accumulation of riboflavin in the "system." The total riboflavin in the "system" is defined by the following equation: $S = T + F + U - D$ where S is total riboflavin in the "system," T is the total tissue riboflavin, F is the total riboflavin in the feces, U is the total riboflavin in the urine and D is the total riboflavin obtained from the diet. Each of the quantities on the right side of the equation was determined by analysis for each animal. Means of the values of S for each animal in the two-, 4-, 6- and 8-week groups of animals were used to give a measure of

[3] Three animals of the 8-week group died of causes unrelated to the deficiency state and were discarded.

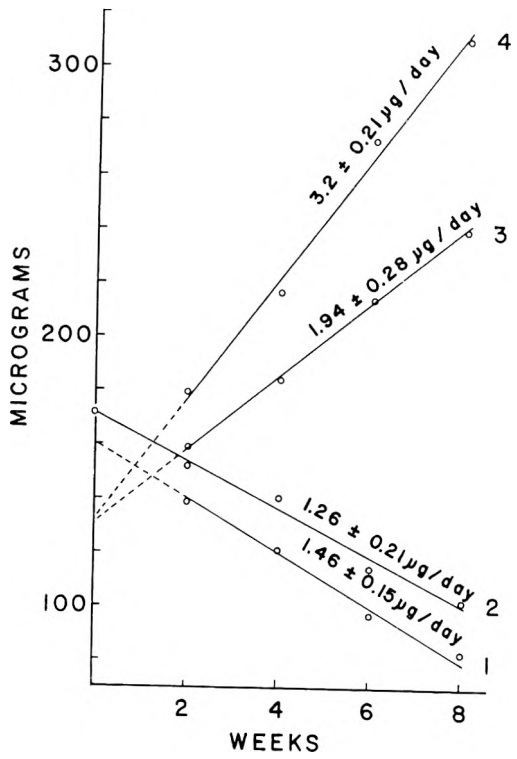

Fig. 1 Mean distribution of riboflavin and C^{14}-riboflavin with best straight line fitted by method of least squares. Curve 1, depletion of C^{14}-riboflavin from tissues, $Y_1 = 160.87 - 10.23$ X. Curve 2, depletion of C^{14}-riboflavin from tissues corrected for urinary riboflavin excretion, $Y_2 = 171.50 - 8.83$ X. Curve 3, accumulation of riboflavin in the "system," $Y_3 = 131.22 + 13.61$ X. Curve 4, total intestinal synthesis of riboflavin, $Y_4 = 132.80 + 22.40$ X.

the accumulation of riboflavin in the "system."

Correction of riboflavin in the "system" for destruction of riboflavin. Using the symbols defined in the two preceding paragraphs S + (I−R) gives a value for riboflavin in the "system" corrected for destruction of riboflavin in the tissues. For purposes of simplicity in calculation of statistical information the equation was rearranged and S−R was calculated for each animal. The regression equation for the variation of (S−R) with time for the two-, 4-, 6- and 8-week groups was derived. To the equation Y = 22.40 X − 40.10 was added a value for the riboflavin-2-C^{14} observed in the initial group, I = 172.90 to give the regression equation 4 of figure 1. This follows, since the equation[4] (S−R) + I is equivalent to S + (I−R) where (I−R) represents riboflavin destroyed in the tissues.

Synthesis of D-*riboflavin-2-*C^{14}. The following synthesis of radioactive riboflavin represents an improvement over that reported from this laboratory on an earlier occasion. Barbituric-2-C^{14} acid, 1.10 gm (Haley and Lambooy, '54), 3.15 gm 1-(D-ribitylamino)-2-phenylazo-4,5-dimethylbenzene,[5] 25 ml of *n*-butyl alcohol and 4 ml of glacial acetic acid were processed by the procedure described by Shunk et al. ('55) for the preparation of 5′-desoxyriboflavin and found to be productive for other 6,7-disubstituted-9-(1′-D-ribityl)isoalloxazines (Lambooy, '58). The purified product weighed 2.12 gm (66% of the theoretical amount based on the barbituric acid used) with a melting point of 296 to 298°C (dec., uncorrected, rapid heating to 250°C), at which point a sample of USP Riboflavin Reference Standard also decomposed. This particular lot of D-riboflavin-2-C^{14} had a specific activity of 1.18 µc/mg or 0.436 mc/mmole.

RESULTS

Accumulation of riboflavin in the "system." The riboflavin content of the feces, urine and carcass less the riboflavin present in the diet were combined for each animal to give the riboflavin in the "system." The mean values of riboflavin in the "system" at each time interval were used for the derivation of a regression equation for the variation of riboflavin accumulation with time by the method of least squares. The slope of this regression equation (line 3, fig. 1) is 1.94 ± 0.28[6] µg per day and represents the rate of accumulation of riboflavin in the "system."

The accumulation of riboflavin in the "system" is linear with time. The close agreement between the slope of the regression equation and the rate of riboflavin

[4] Only the two-, 4-, 6-, and 8-week data were used to derive the regression equations. The "initial" animals provide an independent estimate of the intercept.

[5] The 1-(D-ribityl amino)-2-phenylazo-4,5-dimethyl-benzene was generously supplied by Dr. Karl Pfister of the Merck Sharp and Dohme Research Laboratories.

[6] The ± is an estimate of the standard error.

TABLE 1

Mean distribution of microbiologically active riboflavin per animal

Time	Av. total riboflavin in feces	Rate of fecal excretion of riboflavin	Mean tissue riboflavin	Total urine riboflavin	Tissue riboflavin + urine riboflavin − diet[1] riboflavin
weeks	*μg*	*μg/day*	*μg*	*μg*	*μg*
0			148.6 ± 6.1		
2	17.3 ± 1.6	1.2 ± 0.1	129.5 ± 5.7	13.2 ± 2.3	141.5 ± 6.2
4	41.2 ± 3.5	1.5 ± 0.1	126.9 ± 5.4	19.3 ± 3.5	140.9 ± 5.5
6	74.8 ± 8.4	1.8 ± 0.2	125.4 ± 4.4	17.9 ± 2.9	139.0 ± 3.4
8	105.1 ± 8.2	1.9 ± 0.1	120.6 ± 5.5	19.7 ± 3.1	134.4 ± 4.9

[1] The riboflavin-deficient diet contained 0.02 to 0.03 μg of riboflavin per gm of diet.

excretion in the feces follows from the relative constancy[7] of the mean tissue riboflavin and of the total urine riboflavin (table 1). The decrease in mean tissue riboflavin of only 20% in 8 weeks (table 1) is at variance with the 50% decrease in mean tissue riboflavin in 4 weeks which Bessey et al. ('58) observed for riboflavin-deficient rats.

Rate of destruction of riboflavin. A regression equation was derived for the variation of tissue C^{14}-riboflavin with time. The slope of the equation from figure 1 (line 1) is -1.46 ± 0.15 μg per day. Some of this depletion of labeled riboflavin was due to urinary excretion of riboflavin. A correction for the excretion in the urine was made using values obtained by the microbiological assay. This is justified on the basis of the specificity of the microbiological assay, since the radioactive assay is specific for the C^{14} label only. The corrected value obtained from the slope of the regression equation for the variation of the sum of the mean values for riboflavin in the urine plus C^{14}-riboflavin in the tissues with time is -1.26 ± 0.21 μg per day (line 2, fig. 1). This value represents destruction of riboflavin if the reasonable assumption is made that the label excreted into the gastrointestinal tract (table 2) is not riboflavin. The loss of label from the tissues was accounted for quantitatively by its appearance in the feces and urine (table 2). There is no direct evidence bearing on the excretion of riboflavin into the gastrointestinal tract at physiological levels or lower, but Obermeyer et al. ('45) report that the excretion of riboflavin in the feces is insensitive to the level of riboflavin in the diet, implying complete absorption in the gastrointestinal tract. Furthermore, Bessey et al. ('58) were unable to account for 75% of an unusually large injected dose of 500 μg of riboflavin and the riboflavin not recoverable from the tissues, urine or feces was assumed to be destroyed. Consequently there is little reason to feel that the label in the feces is biologically active riboflavin.

Total rate of synthesis. When the rate of accumulation of riboflavin in the "system" (line 3, fig. 1) is corrected for the rate of destruction of riboflavin in the tissues (line 2, fig. 1), the resulting regression equation that is derived (line 4, fig. 1) has a slope of 3.2 ± 0.21 μg per day. This value represents the total rate of synthesis of riboflavin in the gastrointestinal tract of these riboflavin-deficient rats. It is apparent from the *constant* levels of riboflavin in the tissues that the loss of 1.26 μg per day of riboflavin by destruction is replaced by the absorption of an *equivalent amount* of riboflavin from the *gastrointestinal tract.* The remaining 1.94 μg per day is excreted in the feces.

That more of the riboflavin synthesized is not absorbed may be due to synthesis of riboflavin in regions of the intestine where absorption is limited, or as Bro-Rasmussen ('58) has suggested, tissue protein binding sites for riboflavin may be reduced due to loss of a "labile" tissue protein fraction.

DISCUSSION

Use of the riboflavin-deficient rat offers two important advantages for the measurement of the rate of destruction of riboflavin. The growth rate has ceased and

[7] See footnote 4.

TABLE 2

Mean distribution of C^{14} label per animal and percentage of the total original value (μg per animal)

Group	0	2	4	6	8
weeks					
Tissue	172.9 ± 11.7(100%)	139.1 ± 4.2(80%)	121.5 ± 6.1(71%)	96.7 ± 3.4(56%)	83.1 ± 3.7(48%)
Urine		15.5 ± 1.3(9%)	25.7 ± 2.3(15%)	34.1 ± 2.9(20%)	41.3 ± 3.7(24%)
Feces		13.2 ± 1.1(8%)	20.0 ± 1.2(12%)	27.0 ± 2.1(16%)	34.0 ± 3.0(20%)
Total C^{14}	172.9 ± 11.7(100%)	167.8 ± 4.5(97%)	167.2 ± 6.6(98%)	157.8 ± 4.9(92%)	158.4 ± 6.0(92%)

changes in excretion due to changes in anabolism or catabolism of tissues is circumvented. Secondly, the intake of vitamin from the diet is negligible and the effect of the dietary riboflavin on protein metabolism which secondarily affects the retention of riboflavin is not noted.

The estimate we make for the rate of destruction agrees well with an estimate made by Bessey et al. ('58) of approximately 2 μg per 50 gm of rat per day in the riboflavin-deficient animal. Their estimate is based on the amount of riboflavin that must be supplied to a nongrowing rat to maintain its tissue concentration of riboflavin at a given level. Lambooy ('61), from a plot of log dose of intake of riboflavin versus growth, estimates that about 1.5 μg per day of riboflavin would be required to maintain the deficient animal's weight. This minimal requirement does not support growth, hence it is a measure of rate of destruction. These independent, although indirect, estimates of the rate of destruction give validity to our assumption that fecal excretion of riboflavin derived from the tissues is negligible and that our estimate of the rate of destruction is essentially the correct value.

The question of the nature of the degradation products of riboflavin metabolism is unanswered at present. From the fact that our estimate of labeled material in the urine is higher than the microbiological estimate for riboflavin, it would seem that the degradation products could be demonstrated by chromatographic procedures. A radioautograph of a chromatographed urine sample revealed the presence of riboflavin, and traces of riboflavin phosphate and an unidentified metabolite.

In general the radioactive assays gave a higher value for the initial tissue content of C^{14}-riboflavin, 172.9 ± 11.7 μg, compared with 148.6 ± 6.1 μg for riboflavin by the microbiological assay. Neither of these values, however, affects the slopes of the regression equations since they were not used in their derivation[8] and they merely indicate that the radioactive assay slightly overestimates the

[8] Only the experimental groups were used.

amount of C^{14}-riboflavin present in the initial group.

The total rate of synthesis of 3.2 μg per day is insufficient to meet the animal's requirement for growth. Only 40% of the total that is synthesized appears to be available for use by the animal and of necessity it must be absorbed via the intestinal wall.

SUMMARY

The regression equation for the accumulation of riboflavin in the "system" for the riboflavin-deficient rat, as estimated from the variation of tissue, urine and fecal riboflavin less the riboflavin in the diet with time, showed that the rate of accumulation is 1.94 ± 0.28 μg per day per rat. This is approximately equal to the rate of fecal excretion of riboflavin. The fecal excretion of riboflavin is seen to be superimposed on a relatively constant riboflavin content of the tissues and of constant excretion of riboflavin in the urine.

The rate of destruction of riboflavin was obtained from the slope of the regression equation for the variation of tissue C^{14}-riboflavin with time, and observed to be -1.26 ± 0.21 μg per day per rat.

The correction of the rate of accumulation of riboflavin in the "system" by use of the rate of destruction results in a value for the total rate of synthesis of riboflavin of 3.2 ± 0.21 μg per day in the riboflavin-deficient rat.

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Effect of Mild Chemical and Enzymatic Treatments of Soybean Meal and Soybean Trypsin Inhibitors on Their Nutritive and Biochemical Properties[1]

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The superiority of properly heated soybean meal (HSBM) over that of raw soybean meal (RSBM), as a source of protein for animals, has been observed by numerous investigators (Liener, '58a). The beneficial effect, gained by heat treatment, is a resultant of the destruction of several heat-labile, growth-impairing factors present in soybeans, on the one hand, and of the damage which the protein suffers, on the other. The growth-depressing effect of RSBM has been mainly attributed to an inhibition of intestinal proteolysis, to hemagglutinating activity and also to an inadequate balance of amino acids in the diet (Fisher and Johnson, '58).

Several factors responsible for inhibition of proteolysis have been reported. Kunitz ('46, '47) discovered, purified and characterized a crystalline soybean trypsin inhibitor. Bowman ('46) separated two crude soybean trypsin inhibitors, one of them acetone insoluble (Ai) and the other alcohol insoluble (Ali). Lipke et al. ('54) reported the presence of a fraction C_1 in soybeans, which inhibits trypsin as well as the proteolytic activity of *Tribolium confusum* larvae. Almquist and Merritt ('52a,b, '53) demonstrated that the growth-inhibition of chicks by RSBM, was completely reversed by the addition of crude or crystalline trypsin.

The physiological significance of soybean trypsin inhibitors, pertaining to secretion and fate of pancreatic proteolytic enzymes of rats and chicks, has been investigated by Lepkovsky and associates (Lyman and Lepkovsky, '57; Lepkovsky et al., '59) and by Alumot and Nitsan ('61).

The soybean hemagglutinin was isolated and characterized by Liener and associates (Liener and Pallansch, '52; Liener and Hill, '53; Liener, '53, '58b; Wada et al., '58). Although soybean hemagglutinin does not inhibit proteolysis, it is responsible, according to Liener ('53), for about 50% of the growth inhibition of rats fed RSBM.

The present investigation was initiated to find out whether mild chemical or enzymatic treatments of RSBM could be used to remove or inactivate the deleterious factors, without adversely affecting the nutritional value of the soybean protein. Evans ('46) demonstrated in *in vitro* experiments that RSBM, predigested by pepsin, was hydrolyzed by trypsin to the same extent as HSBM. Kassel and Laskowski ('56) reported that the crystalline soybean trypsin inhibitor is completely inactivated by pepsin and undergoes a reversible inactivation when treated with HCl (pH 1.6). Borchers et al. ('47) noted that RSBM, when extracted with dilute acid at pH 4.2, leaves an insoluble residue, of a nutritional value, for chicks, similar to that of HSBM. The present study was designed to study the growth effect of RSBM residue insoluble at pH 4.2 on chicks, rats and *Tribolium castaneum* larvae, as the latter showed a correlation to chicks, when grown on commercially treated, soybean meals (SBM) (Birk and Applebaum, '60).

Another aim of this work was to examine the stability of crude soybean trypsin inhibitors and hemagglutinating activity towards dilute HCl and peptic digestion — the natural precursors of intestinal proteolysis.

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MATERIALS AND METHODS

Soybean meals and soybean trypsin inhibitors. Raw soybean meal (RSBM) was prepared by finely milling commercial, defatted soybean flakes.[2] The nitrogen content of RSBM was 8.2%.

Soybean meal insoluble at pH 4.2 ($SBM_{4.2}$) was prepared by removing the fraction soluble at that pH. A 1:10 suspension of RSBM in 0.05 N HCl was stirred for one hour at room temperature and then suction-filtered. The precipitate was oven-dried at 38 to 40°C and then ground. The resulting yield of $SBM_{4.2}$ was 73% and its nitrogen content, 9.9%.

The soybean meal fraction soluble at pH 4.2 ($F_{4.2}$) was prepared by adding 9 volumes of acetone to $SBM_{4.2}$ filtrate. The acetone precipitate was allowed to settle overnight and was then separated by centrifugation, dried in vacuum and finely ground; dried $F_{4.2}$ was obtained in a yield of 10% from the original RSBM. The nitrogen content of dried $F_{4.2}$ was 5.9%.

Properly heated soybean meal (HSBM) was a commercial product[3] with a nitrogen content of 7.5%.

Three crude soybean trypsin inhibitors were prepared from RSBM. Ai, acetone-insoluble and Ali, alcohol-insoluble fractions, were prepared according to Bowman ('46), omitting the 2.5% TCA 95°C treatment for Ai. C_1, a fraction from soybeans that inhibits trypsin and the proteolytic activity of *Tribolium confusum* larval midgut homogenate was prepared according to Lipke et al. ('54). The nitrogen content of Ai, Ali and C_1 was determined at 1.5, 9.1 and 14.4%, respectively.

Determination of trypsin-inhibiting and hemagglutinating activities. Trypsin-inhibiting activity was determined by the casein digestion method of Kunitz ('47), as described by Laskowski ('55) at 280 mμ with a Beckman Spectrophotometer, model DU, using crystalline salt-free trypsin,[4] vitamin-free casein[5] and Sørensen 0.1 M phosphate buffer (pH 7.6) prepared from $Na_2HPO_4 \cdot 12\ H_2O$[6] and $NaH_2PO_4 \cdot 2\ H_2O$[7] according to Gomori ('55). A 0.5-ml solution of inhibitor in buffer, replacing 0.5 ml of buffer in the reaction mixture, was pre-incubated together with the trypsin solution for 30 minutes at 37°C and the reaction was then started by addition of casein.

The trypsin inhibitor units (TIU) (Kunitz, '47) were expressed, in terms of proteolytic units inhibited, the proteolytic unit being defined as the increase of one unit of optical density at 280 mμ per minute of digestion under optimal experimental conditions.

Hemagglutinating activity was determined by the photometric method developed by Liener ('55).

TIU and hemagglutinating units (HU) were calculated per mg of the following: protein (N × 6.25), SBM or inhibitor.

Feeding trials with T. castaneum larvae, rats and chicks. *T. castaneum* larval growth experiments were conducted according to Birk and Applebaum ('60) with diets consistng of 5% of dried yeast,[8] SBM and wheat flour in amounts that provided equal nitrogen content in the various diets. Percentages of SBM and wheat flour in the three experimental diets were as follows:

Diet no. 1: 65.5% wheat flour; 29.5% RSBM
Diet no. 2: 70.5% wheat flour; 24.5% $SBM_{4.2}$
Diet no. 3: 63.5% wheat flour; 31.5% HSBM.

Each diet was assayed in 5 replicates consisting of 10 larvae per replicate. After 18 days the larvae were removed from their diets, each group was weighed and returned to the vial. Age to pupation was determined by daily observations of each vial and ET_{50} (days from beginning of experiment to pupation of 50% of the population) was calculated.

Weanling male rats of an inbred Wistar strain, 24 to 27 days old, were maintained for 4 weeks with diets containing 10% of protein (N × 6.25) supplied by the three tested soybean meals (RSBM, $SBM_{4.2}$ and HSBM) as the only protein source. The composition of the diets used is given in

[2] The defatted soybean flakes, a gift of Izhar Oil Industry of Israel Ltd., were prepared by dehulling and flaking the soybeans at 70 to 80°C for 20 minutes, defatting the flakes with petrol-ether 50°C for three hours and evaporating the solvent at room temperature.

[3] Prepared at Izhar Oil Industry of Israel Ltd., by heating the defatted flakes at 80 to 100°C for 20 minutes, steaming for 25 minutes at 118°C and drying at room temperature.

[4] Worthington Biochemical Corporation, Freehold, New Jersey.

[5] Nutritional Biochemicals Corporation, Cleveland.

[6] BDH Analar.

[7] See footnote 6.

[8] Hopkin and Williams, Ltd., England.

table 1. Twelve rats were used for each feed treatment. They were housed in groups of 6, in cages with screen floors in a room maintained at 25°C, and consumed food and water ad libitum. The rats were weighed individually at the end of each week.

An additional experiment was performed with 5 groups of rats, each group consisting of 6 rats grown for three weeks with diets containing the soybean meals assayed. Groups 1–3 were fed diets including RSBM, $SBM_{4.2}$ and HSBM, respectively, as described in table 1. The diets of groups 4 and 5 were essentially the same as those of groups 2 and 3, but 2.2% of corn flour were replaced by 2.2% of $F_{4.2}$, corresponding to the yield of $F_{4.2}$ when extracted from RSBM. The rats were weighed individually starting with the third week of the experiment.

Day-old male White Leghorn chicks were distributed, on a weight basis, into 9 groups of 20 and grown with an all-vegetable diet, the main protein source being the three experimental soybean meals. Groups 1–3 were fed diets including RSBM, $SBM_{4.2}$ and HSBM, respectively (see table 2). Diets for groups 4–6 and 7–9 were essentially the same as those for groups 1–3, but were supplemented with 0.3 and 0.6% of DL-methionine,[9] respectively. Each group was weighed at the end of each week and the amount of ingested food was determined.

TABLE 1

Composition of experimental diets for rats

	%
Dextrose[1]	36.0
Soybean oil	5.0
Salt-vitamin mixture no. 1[2]	1.0
Salt mixture no. 2 (USP)[3]	3.0
Soybean protein source:[4]	
RSBM	19.6
or $SBM_{4.2}$	16.3
or HSBM	21.0
Corn flour to total 100	+
Vitamins A and D[5]	

[1] J. T. Baker Chemical Company, Phillipsburg, New Jersey.

[2] Composition in per cent: thiamine·HCl, 0.03; riboflavin, 0.03; pyridoxine·HCl, 0.03; niacin, 0.05; Ca pantothenate, 0.2; choline chloride, 10; salt mixture no. 2 to total 100.

[3] Nutritional Biochemicals Corporation, Cleveland.

[4] To give 10% (N × 6.25) protein.

[5] Supplied once a week as cod liver oil.

TABLE 2

Composition of experimental diets for chicks

	%
Alfalfa meal, dehydrated[1]	2.5
Dicalcium phosphate	2.0
Ground oyster shells	2.0
Mineral concentrate[2]	0.4
Vitamin concentrate[3]	0.5
Antibiotic supplement[4]	0.1
Coccidiostat supplement[5]	0.1
Soybean protein source:[6]	
RSBM	30.2
or $SBM_{4.2}$	23.1
or HSBM	31.3
Ground sorghum meal[7] to total 100	+

[1] Contained 20% protein (N × 6.25).

[2] Composition/100 gm salt mixture: (in grams) sodium chloride, 94.7; manganese sulfate, 2.6; iodine, 0.048; iron, 0.14; copper, 0.1; cobalt, 0.008; zinc, 0.07; molybdenum, 0.0044; inactive ingredients, 2.4.

[3] Composition/100 gm vitamin concentrate: vitamin A, 300,000 IU; vitamin D_3, 40,000 IU; vitamin E, 40 IU; riboflavin, 160 mg; Ca pantothenate, 320 mg; niacin, 800 mg; choline chloride, 6 gm; vitamin K, 80 mg; vitamin B_{12}, 0.4 mg; BHT, 5 gm.

[4] Aurofac 20, Agricultural Department, Cyanamid International, New York.

[5] Bifuron K, Abic Ltd., Israel.

[6] To give approximately 15% soy protein (N × 6.25).

[7] Contained 11% protein (N × 6.25).

The trial was terminated after 4 weeks and the chicks were then weighed individually.

HCl and pepsin-treated RSBM, $SBM_{4.2}$, Ai, Ali, C_1 and $F_{4.2}$. Suspensions of 10 gm of RSBM in 100 ml of HCl (pH 1.6), containing zero to 2% of pepsin[10] (1–10,000) were incubated for 48 hours at 37°C. Ten grams of RSBM suspended in 100 ml of 0.1 M phosphate buffer (pH 7.6) served as the control. Five-milliliter samples were taken out from each reaction flask after various incubation periods. The samples were centrifuged for 15 minutes at 1600 × g. Aliquots of the supernatants were neutralized and diluted with 0.1 M phosphate buffer (pH 7.6) or saline and then assayed for trypsin-inhibiting and hemagglutinating activities, respectively. The residues were further incubated for 6 hours at 37°C with 0.1 M phosphate buffer (pH 7.6) (volume of buffer being equal to the supernatant removed in each case) and then centrifuged. The pH 7.6 supernatants were assayed as described.

[9] N. Preminger Ltd., Israel.

[10] See footnote 5.

Comparative zero-, 2-, 4- and 6-hour treatments of RSBM and $SBM_{4.2}$ with dilute HCl and with 0.2% of pepsin were performed by suspending 10% RSBM or 8.2% $SBM_{4.2}$ (on the basis of a 5% protein concentration) in HCl (pH 1.6) containing 0.2% of pepsin or none. After incubation was terminated the samples were assayed for trypsin-inhibiting activity as described above.

HCl and pepsin-treated Ai, Ali, C_1 and $F_{4.2}$ were prepared by dissolving 2% of each in dilute HCl solutions containing zero, 0.02 and 0.2% of pepsin and adjusting the pH to 1.6. Because of the high nitrogen content of C_1 this factor was also incubated with 2% of pepsin. Two per cent solutions of the inhibitors in 0.1 M phosphate buffer (pH 7.6) served as controls. The reaction mixtures were incubated for 48 hours at 37°C and then neutralized and diluted with 0.1 M phosphate buffer (pH 7.6) or saline. Samples of each reaction mixture were assayed for trypsin-inhibiting and hemagglutinating activities respectively.

RESULTS

Comparative properties of RSBM, $SBM_{4.2}$, HSBM and $F_{4.2}$

Trypsin-inhibiting and hemagglutinating activities of RSBM, $SBM_{4.2}$, HSBM and $F_{4.2}$, dissolved in 0.1 M phosphate buffer (pH 7.6) and saline, respectively, and immediately assayed, are shown in table 3.

TABLE 3

Trypsin-inhibiting and hemagglutinating activities of RSBM, $SBM_{4.2}$, HSBM and $F_{4.2}$

Material examined	Activity/mg protein (N × 6.25)	
	TIU[1] × 10^{-3}	HU[2]
RSBM	84.2	107.0
$SBM_{4.2}$	43.1	8.4
HSBM	2.1	4.5
$F_{4.2}$	400.0	649.0

[1] Trypsin-inhibitor units.
[2] Hemagglutinin units.

$SBM_{4.2}$, the residue of RSBM after extraction with dilute HCl, retains only 7 and 50%, respectively, of the original hemagglutinating and trypsin-inhibiting activities of RSBM, on a protein basis. The lower-than-expected yield of $F_{4.2}$ hemagglutinating activity is probably due to a loss in activity as a result of its sensitivity to the preparation process.

The nutritional values for T. castaneum larvae, rats and chicks of RSBM, $SBM_{4.2}$, HSBM and $F_{4.2}$ were determined by growth experiments. The growth response of the above-mentioned organisms to the three soybean meals, which served as the main or only protein source, is summarized in table 4. The results presented in this table

TABLE 4

Effect of RSBM, $SBM_{4.2}$ and HSBM on growth of T. castaneum larvae, rats and chicks

SBM assayed	*T. castaneum* larvae		Rats	Chicks					
				A[4]		B[5]		C[6]	
	Average weight of larva[1]	ET_{50}[2]	Weight gain[3]	Average[3] weight of chick	Gm gain/ gm food	Average[3] weight of chick	Gm gain/ gm food	Average[3] weight of chick	Gm gain/ gm food
	mg		*gm*	*gm*		*gm*		*gm*	
RSBM	1.08	26.6	−0.4	153.2	0.44	178.9	0.38	187.4	0.39
$SBM_{4.2}$	1.63	23.0	9.8	170.4	0.38	214.8	0.45	205.1	0.44
HSBM	2.30	20.2	54.1	242.5	0.46	275.9	0.49	260.6	0.50
L.S.D. at $P = 0.05$	0.15	1.5	7.4			14.8[7]			
L.S.D. at $P = 0.01$	0.21	2.2	9.9			20.0[7]			

[1] After 18 days from start of experiment.
[2] Days from start of experiment to pupation of 50% population.
[3] After 28 days from start of experiment.
[4] A indicates no methionine added.
[5] B indicates 0.3% methionine-supplemented diet.
[6] C indicates 0.6% methionine-supplemented diet.
[7] Calculated for main effects (of average weights) only.

show also the effect of methionine on the growth of chicks fed methionine-supplemented experimental diets. As no interaction has been found, statistically, between the soybean meals assayed and the level of methionine added, the least significant differences were calculated for the main effects only, i.e., for the three different SBMs and for the three methionine levels.

Highly significant differences ($P < 0.01$) have been found between the three soybean meals as well as between the methionine-supplemented and nonsupplemented diets. No significant difference was found between the two methionine levels of supplementation.

The effect of $F_{4.2}$ on the growth of rats, when added to $SBM_{4.2}$ and HSBM diets is shown in table 5.

The growth impairment caused by $F_{4.2}$, when added to HSBM, amounts only to 28% although this fraction contains most of the hemagglutinating and about 50% of the trypsin-inhibiting activities.

TABLE 5

Effect of $F_{4.2}$ supplemented $SBM_{4.2}$ and HSBM on growth of rats

SBM assayed	Weight gain[1]
	gm
RSBM	0.7
$SBM_{4.2}$	7.2
$SBM_{4.2}$ + 2.2% $F_{4.2}$[2]	0.3
HSBM	35.7
HSBM + 2.2% $F_{4.2}$[2]	25.9
L.S.D. at $P = 0.05$	6.7
L.S.D. at $P = 0.01$	9.1

[1] After 21 days from start of experiment.
[2] Percentage based on diet.

Effect of HCl (pH 1.6) and peptic digestion on the trypsin-inhibiting and hemagglutinating activities of RSBM, $SBM_{4.2}$, Ai, Ali, C_1 and $F_{4.2}$

The remaining hemagglutinating and trypsin-inhibiting activities of RSBM, after being treated with HCl (pH 1.6) and with various concentrations of pepsin for one to 48 hours, is presented in table 6.

TABLE 6

Effect of HCl (pH 1.6) and peptic digestion on the trypsin-inhibiting and hemagglutinating activity of RSBM

10% RSBM suspended in:	Activity/mg RSBM									
	Period of incubation in hours									
	1		4		8		24		48	
	TIU[1] × 10^{-3}	HU[2]	TIU × 10^{-3}	HU	TIU × 10^{-3}	HU	TIU × 10^{-3}	HU	TIU × 10^{-3}	HU
HCl (pH 1.6)	43.5	21.6	31.6	16.0	25.1	9.8	19.8	3.0	12.8	0.2
HCl (pH 1.6) containing 0.1% pepsin	42.6	19.0	22.2	3.2	17.6	1.5	13.4	0.7	8.5	0.1
HCl (pH 1.6) containing 0.5% pepsin	40.8	10.9	18.5	0.1	14.5	0.1	10.7	none	5.7	none
HCl (pH 1.6) containing 1.0% pepsin	40.4	2.7	17.7	none	12.2	none	8.5	none	4.0	none
HCl (pH 1.6) containing 2.0% pepsin	39.4	none	15.5	none	11.5	none	4.2	none	3.0	none
0.1 M phosphate buffer (pH 7.6)	46.0	52.7	44.8	52.7	44.8	52.6	38.0	52.8	37.0	53.0

[1] Trypsin inhibitor units.
[2] Hemagglutinin units.

From the results presented in table 6 it is clear that the trypsin-inhibiting and hemagglutinating activities are strongly affected both by dilute HCl and by pepsin. One hour's incubation of RSBM in HCl (pH 1.6) results in a decrease of 60% in hemagglutinating activity; 4 hours' incubation, under the same conditions, decreases the trypsin-inhibiting activity by 30% and the hemagglutinating activity by 70%. After 4 hours' digestion by 0.5% pepsin, RSBM still retains 41% of its trypsin-inhibiting activity but loses almost completely its hemagglutinating power. It may thus be concluded that both pepsin concentration and time of incubation play an important role in the inactivation of these two detrimental activities. As the RSBM residues from the various 48-hour incubations, when incubated for additional 6 hours in 0.1 M phosphate buffer, showed no hemagglutinating and only slight trypsin-inhibiting activity, it may be concluded that these two deleterious activities are inactivated irreversibly by HCl (pH 1.6) and pepsin.

The results presented in tables 4 and 5 clearly indicate that $SBM_{4.2}$ retains about 50% of the original trypsin-inhibiting activity of RSBM and almost no hemagglutinating activity and yet highly impairs the growth of rats and chicks. An attempt was therefore made to clarify further the detrimental activity of $SBM_{4.2}$ by examining the susceptibility of its trypsin-inhibiting activity to HCl and pepsin as compared with that of RSBM. The comparative decrease of trypsin-inhibiting activity of $SBM_{4.2}$ and RSBM is illustrated in figure 1.

The trypsin-inhibiting activity of $SBM_{4.2}$ decreased by a smaller extent than that of RSBM when incubated with HCl (pH 1.6) and with pepsin under the same experimental conditions (fig. 1).

To further elucidate the relative role of various soybean trypsin-inhibiting fractions in the low nutritional value of RSBM, the factors Ai, Ali, C_1 and $F_{4.2}$ were studied. The trypsin inhibitor units (TIU) and hemagglutinin units (HU) per milligram of weight of examined material (dissolved in 0.1 M phosphate buffer, pH 7.6, and saline, respectively) were determined as follows: 80×10^{-3} TIU and no HU for Ai; 135×10^{-3} TIU and 20.2 HU for Ali;

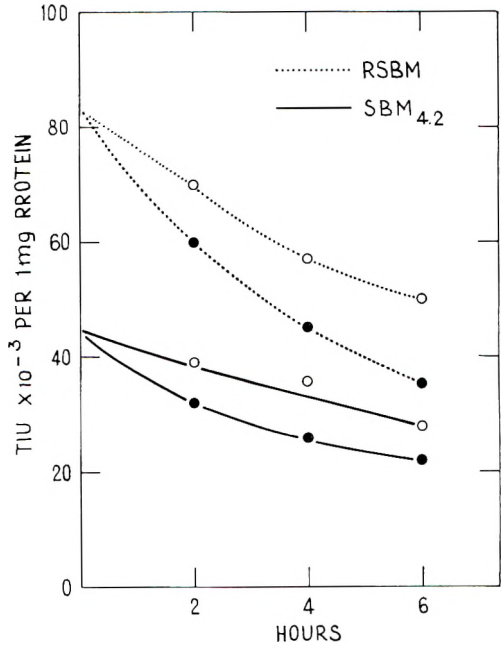

Fig. 1 Comparative decrease in trypsin-inhibiting activity of RSBM and $SBM_{4.2}$ treated with HCl (pH 1.6) and with pepsin: RSBM incubated with HCl (pH 1.6) (···○···), RSBM incubated with HCl (pH 1.6) containing 0.2% pepsin (···●···), $SBM_{4.2}$ incubated with HCl (pH 1.6) (——○——) and $SBM_{4.2}$ incubated with HCl (pH 1.6) containing 0.2% pepsin (——●——).

1082×10^{-3} TIU and 204 HU for C_1 and 147.5×10^{-3} TIU and 240 HU for $F_{4.2}$. In figure 2 is illustrated the relative inactivation of trypsin-inhibiting and hemagglutinating activities of Ai, C_1, Ali and $F_{4.2}$ after 48 hours' incubation with HCl (pH 1.6) and with pepsin. Inactivation is expressed in percentage of decrease of activity by subtracting the remaining TIUs and HUs from the corresponding original activity units of the assayed materials.

The high sensitivity of hemagglutinating activity of all examined materials to HCl (pH 1.6) and to pepsin is illustrated in figure 2. The sensitivity of trypsin-inhibiting activity, on the other hand, varies with the different materials: whereas Ali and $F_{4.2}$ are inactivated, to a different extent, both by HCl (pH 1.6) and pepsin, Ai and C_1 are quite resistant to HCl but are inactivated to a considerable extent, by pepsin depending on its concentration.

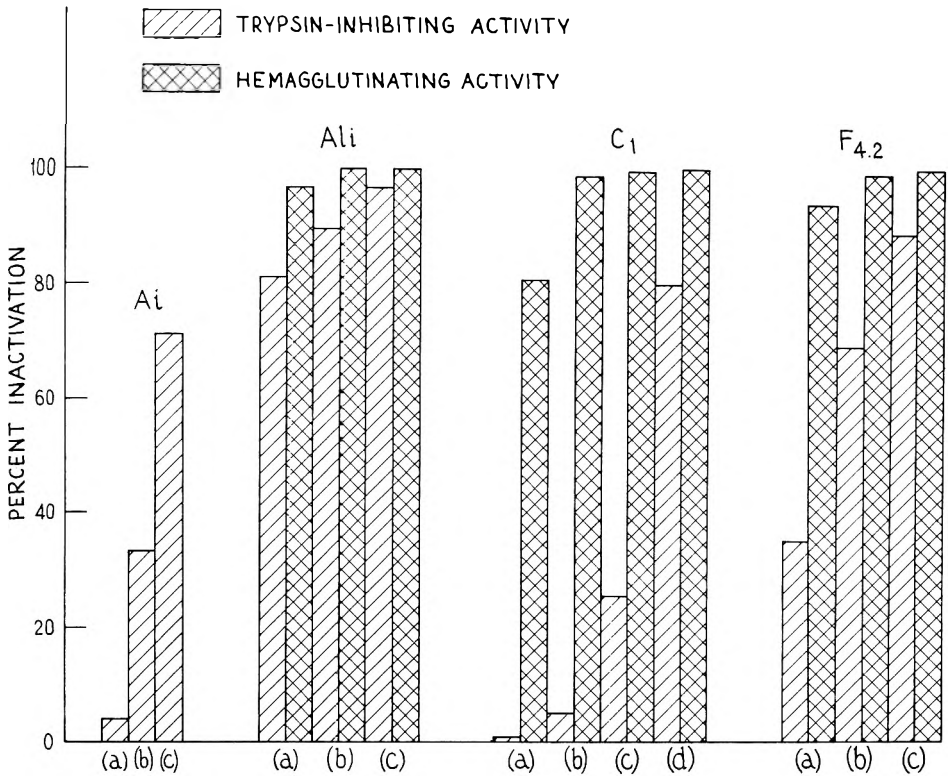

Fig. 2 Inactivation of trypsin-inhibiting and hemagglutinating activities of Ai, C_1, Ali and $F_{4.2}$ after 48 hours incubation with: (a) HCl (pH 1.6); (b) HCl (pH 1.6) containing 0.02% pepsin; (c) HCl (pH 1.6) containing 0.2% pepsin; and (d) HCl (pH 1.6) containing 2% pepsin.

DISCUSSION

It could be expected, from *in vitro* determinations of trypsin-inhibiting and hemagglutinating activities of RSBM, $SBM_{4.2}$ and $F_{4.2}$ (table 3), that the nutritional value of $SBM_{4.2}$ would be comparatively high. A previous report by Borchers et al. ('47) claimed that the nutritional value, for chicks, of the insoluble residue of RSBM extracted at pH 4.2 is similar to that of properly heated SBM. However, comparative *in vivo* trials with chicks, rats and *T. castaneum* larvae showed (table 4) that $SBM_{4.2}$ had but a slightly higher nutritional value than RSBM for chicks and rats, although it retained only slight hemagglutinating activity and about 50% of the original trypsin-inhibiting activity of RSBM; growth response of *T. castaneum* larvae was in accord with the trypsin-inhibiting evaluation *in vitro*. It is suggested that the higher nutritional value of $SBM_{4.2}$, as described by Borchers et al. ('47), should be attributed to the comparatively higher protein content of the acid-extracted SBM diet, as the soybean meals were included in these diets on a weight basis and the protein content of acid-extracted SBM is higher than that of HSBM.

It may be inferred from table 5 that $F_{4.2}$, when added to a HSBM diet, inhibits the growth of rats to the extent of one-third of growth inhibition caused by the $SBM_{4.2}$ diet, although both diets have almost the same TIU content and the $F_{4.2}$-supplemented HSBM diet contains 7 times more HU than the $SBM_{4.2}$ diet. It may thus be concluded that the growth impairment of chicks and rats by RSBM is due only to a small extent to its hemagglutinating activity.

The assumption that hemagglutinating activity plays only a minor role in the detrimental influence *in vivo* of RSBM is

also substantiated by the *in vitro* treatment with dilute HCl and pepsin. The hemagglutinating activity of RSBM and of various fractions prepared from it is very sensitive to HCl (pH 1.6) and to peptic digestion — the natural precursors of pancreatic digestion (table 6 and fig. 1).

Dilute HCl and pepsin also affect the trypsin-inhibiting activity of the various factors, but to a varying extent. It seems (fig. 1) that the lower than expected nutritional value of $SBM_{4.2}$ is at least partly due to the relatively higher stability of the trypsin-inhibiting factors present in $SBM_{4.2}$ towards dilute HCl and pepsin. It might well be that some other thermolabile factors present in $SBM_{4.2}$ are participating in its growth-inhibiting activity.

The fact that in growth experiments with chicks (table 4) no interaction was found between the various soybean meals and the level of methionine added indicates that methionine availability is not responsible for the differences in nutritional values of RSBM, $SBM_{4.2}$ and HSBM.

SUMMARY

Raw soybean meal (RSBM) was extracted with HCl (pH 4.2) yielding an insoluble residue ($SBM_{4.2}$) with a higher protein content. Although $SBM_{4.2}$ retained about 50% of the original RSBM trypsin-inhibiting activity and almost no hemagglutinating activity, it was only slightly superior to RSBM when added to diets of chicks and rats.

Fraction $F_{4.2}$, the acetone precipitated and vacuum-dried extract (pH 4.2) of RSBM, when added to a properly heated soybean meal diet, impaired growth only to the extent of one-third of the growth-impairment caused by $SBM_{4.2}$ although $F_{1.2}$ contains about 50% of trypsin-inhibiting and most of the hemagglutinating activities of RSBM.

The stability of the trypsin-inhibiting and hemagglutinating activities of RSBM, $SBM_{4.2}$ and three crude soybean trypsin inhibitors towards HCl (pH 1.6) and peptic digestion were examined in an attempt to elucidate the fate of the two detrimental activities prior to pancreatic digestion. Analyses of residual trypsin-inhibiting and hemagglutinating activities showed that hemagglutinating activity was completely destroyed even when incubated with HCl alone, whereas trypsin-inhibiting activity was destroyed to a varying extent, depending upon the material examined. The trypsin-inhibiting activity of $SBM_{4.2}$ was found relatively more resistant towards HCl and pepsin, thus leading to the conclusion that its higher than expected growth-depressing activity might be due partly to the presence of a comparatively more resistant trypsin-inhibiting activity.

Growth experiments with chicks maintained with diets including RSBM, $SBM_{4.2}$ and properly heated soybean meal supplemented by various methionine levels showed no interaction between the various soybean meals and the level of methionine added.

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Hydrogenated Fats in the Diet and Lipids in the Serum of Man[1]

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In man, the average cholesterol response in the serum to changes in the dietary fat can be predicted reasonably well when only the common natural fatty acids are involved (Keys et al., '57, '59; Turpeinen et al., '60). Whether the same relationships hold with the unnatural fatty acids produced by hydrogenation is uncertain.

Bronte-Stewart et al. ('56) reported that the cholesterol-depressing effect of highly unsaturated food oils is lost after hydrogenation and Ahrens et al. ('57) noted generally higher cholesterol levels in the serum when such oils were replaced in the diet by the hydrogenated product of the same oils. Similarly, Malmros and Wigand ('57) reported that the cholesterol-depressing effect of whale oil was abolished by hydrogenation. But Beveridge et al. ('58) failed to find, in a brief experiment, a difference in serum cholesterol effect between natural and hydrogenated corn oil.

On superficial consideration, two other sets of observations suggest discrepancies. Malmros ('58) reported that the cholesterol-depressing effect of corn oil is not completely abolished by hydrogenation which reduced the diene content from 50% to only 2%. This would be understandable, however, if, as was almost certainly the case, the saturated fatty acid content of the diet containing the hydrogenated material was less than that of the previous "free" diet. Beveridge and Connell ('60) substituted different commercial margarines for carbohydrates amounting to 45% of total calories in a basic fat-free diet and observed in 6 cases no significant change and in only two an increase in the concentration of cholesterol in the serum. This would be understandable, too, if the margarines contained only a low proportion of fully saturated fatty acids.

Examination of previous reports leads to the conclusion that more systematic experiments, with dietary fatty acids specified in greater detail, are necessary to allow more precise conclusions. The present paper reports experiments in which attempts were made to meet these requirements.

GENERAL PROCEDURE

Three experiments, each involving from 23 to 27 men, aged 39 to 66, were performed under the controlled conditions of the closed metabolic unit of the Hastings State Hospital. The subjects were patients with stabilized schizophrenia who were in good physical health and whose physical and laboratory examinations disclosed no abnormalities.

After a preliminary period of consuming a "controlled house diet" corresponding to a typical American diet, in experiments K and N the men were assigned to one of two or one of 4 groups matched with respect to age and relative body weight. In experiment AD the serum cholesterol level with a controlled diet was also considered in matching the groups.

Successive dietary periods of 21 days each were used as indicated in the experimental designs given in table 1. These designs automatically compensate for any general time trends that may have occurred.

Body weight in night clothing was recorded twice weekly. Venous blood samples were drawn from each man before breakfast on two of the last three days of each dietary period. The analyses for cholesterol, phospholipds and triglycerides in

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TABLE 1

Designs for experiments K, N, and AD (diets indicated were fed for successive 21-day periods to groups of men matched at the outset)

Exp.	No. men	Period 1	Period 2	Period 3	Period 4	Period 5
K	14	House[1]	HS	S	House	—
K	13	House	S	HS	House	—
N	6	House	SF	HSF	House	—
N	6	House	HSF	SF	House	—
N	7	House	C	HC	House	—
N	7	House	HC	C	House	—
AD	6	House	HCO	M	B	House
AD	6	House	M	HCO	B	House
AD	5	House	B	M	HCO	House
AD	6	House	B	HCO	M	House

[1] "House" indicates a standardized version of the hospital diet, controlled as to amount of each food item for each man.

the serum were made by the methods described elsewhere (Keys et al., '60; Grande et al., '61).

DIETS

In three separate experiments 9 diets were used. In all experiments the nutrients in standard servings (weight or volume) of each food were calculated from analytical data on the fats used and from tables of food composition, mainly U. S. Department of Agriculture Handbook no. 8 (Watt and Merrill, '50). In order to maintain body weight constant, adjustments were made in the servings for individual men. On every third day the food rejections and plate waste were recorded for each subject.

In experiment K, 30 gm of experimental fat were substituted for 68 gm of carbohydrate in the controlled house diet, the experimental fat being either natural[2] or selectively hydrogenated safflower oil, with the composition summarized in table 2. The controlled house diet, without modification, corresponds to a typical American diet, providing about 40% of the total calories from fats and being generous in meat and dairy products.

TABLE 2

Composition of the experimental fats in experiments K and N[1] (fatty acids as percentage of total fatty acids)

Fat	Saturated	Monoene	Polyene
	%	%	%
Safflower oil	12	10	78
Hydrogenated safflower oil	32	55	13
Corn oil	15	27	58
Hydrogenated corn oil	26	68	6

[1] Analyzed by alkali isomerization by the Northern Regional Research Laboratory and by the Miami Valley Research Laboratory of Procter and Gamble Company and confirmed by gas-liquid chromatography in this laboratory.

Experiment N involved a single low-fat base diet providing 2310 Cal., 100 gm of protein and 39 gm of fat, including 12 gm of butterfat, 4 gm of fats from vegetable sources, the remaining 23 gm of fat being provided mainly from beef and pork. To this low-fat base diet, 100 gm of one or another of 4 fat supplements were added, the natural or hydrogenated safflower oil used in experiment K, or natural[3] or selectively hydrogenated corn oil, with the composition shown in table 2. Estimates of *trans* fatty acid content expressed as equivalent elaidic acid, determined in two laboratories by infrared absorption, were 33 and 37% for hydrogenated safflower oil and 37% for hydrogenated corn oil.

The average daily nutrients actually eaten in experiments K and N are shown in table 3. The fatty acid compositions of the house diet and of the low-fat base diet food items were calculated from tables of food composition, mainly given by Hilditch ('56). Since these items were constant in each experiment, errors in these estimates

[2] Supplied by Pacific Vegetable Oil Corporation, courtesy of R. W. Hammond.

[3] Donated by Corn Products Company, courtesy of Dr. D. M. Rathmann.

TABLE 3

Nutrients eaten daily in experiments K and N

Exp.	Diet	Total Cal.[1]	% of total calories[2]			
			Total fat	S	M	P
K	Safflower oil, 30 gm	3278	46.0	19.8	17.6	8.6
K	Hydrogenated safflower oil, 30 gm	3240	46.0	21.5	21.3	3.2
N	Safflower oil, 100 gm	3294	37.7	8.5	7.4	21.8
N	Hydrogenated safflower oil, 100 gm	3232	38.3	14.2	20.0	4.1
N	Corn oil, 100 gm	3164	38.9	9.6	12.4	16.9
N	Hydrogenated corn oil, 100 gm	3190	38.5	12.6	23.7	2.2

[1] The nutrients provided by the fat supplements were calculated from analysis, those from the rest of the foods were based on food tables.

[2] The caloric value of total fat was taken as 9 Cal./gm and the total fat calories were divided into S, M and P, respectively, in proportion to the amount of saturated, monoene and polyene in the total fatty acids.

TABLE 4

Composition of experimental fat supplements in experiment AD (fatty acids in percentage of total fatty acids)

Fatty acid	Supplements		
	HCO	M	B
	Selectively[1] hydrogenated corn oil	Mixture	Butterfat
Saturated	18.7	19.6	60.3
Monoene			
cis	20.1	56.8	33.7
trans	36.1	0	0
Total monoene	56.2	56.8	33.7
Diene			
Nonconjugated			
cis, cis	12.8	22.9	4.1
cis, trans	2.0	0	0
trans, trans	5.5	0	0
Conjugated			
cis, trans	1.7	0	0
trans, trans	3.1	0	0
Total diene	25.1	22.9	4.1
Triene	0	0.7	1.9
Total polyene	25.1	23.6	6.0

[1] Fatty acid compositions were mean values of 4 analyses by gas-liquid chromatography. The infrared analysis was done by F. H. Fryer, Standard Brands Incoporated, Indianapolis, Indiana, who reported absorption at 10.36 microns indicated 50.3% isolated *trans* double bonds (as elaidic acid) and absorption at 3.28 microns indicated 48.6% isolated *cis* double bonds (as oleic acid).

are unimportant; only the fat supplements were varied.

The three diets of experiment AD were made up of the same low-fat base diet used in experiment N plus supplements of experimental fats. Supplement HCO was 98.5 gm of selectively hydrogenated corn oil[4] (a different batch than used in experiment N). Supplement M was 100 gm of a mixture of 21.4 gm of this same corn oil[4] before hydrogenation, 77.9 gm of olive oil and 0.7 gm of totally hydrogenated corn oil. Supplement B was 101.2 gm of butterfat. The fatty acid composition of these fat supplements was estimated by gas-liquid chromatography using a butanediol succinic acid polyester column (Craig and Murty, '58) and the findings are summarized in table 4.

In experiment AD, 7-day composites of one standard serving of the diet with the fat supplement M were collected on 4 occasions. The composites were homogenized in a gallon-size Waring Blendor and analyzed (Grande et al., '61). From the means of these analyses and the data on fat supplement M, the composition of the low-fat base diet was estimated to be, in grams daily, dry matter, 473.8; ash, 18.4; fat, 33.7; protein, 8.14; and carbohydrate (by difference), 340.3, with total Cal., 1991. Assuming for the purpose of computation that the fat contained only glycerides, the data from gas-liquid chromatography indicated that the composition of the daily serving of the low-fat base diet contained 17.4 gm of saturated, 13.1 gm of monoene, 2.2 gm of diene and 0.9 gm of triene fatty acid glycerides.

In experiment AD, as in experiments K and N, corrections for calorie adjustments to maintain balance and for rejections and plate waste were made. The average for

[4] Prepared especially and donated by Standard Brands Incorporated, courtesy of Mr. G. B. Crump.

these corrections amounted to − 4.6 gm of protein, — 1.5 gm of fat and — 126 Cal. Conceivably, these estimated corrections might be in error by as much as 25 or even 30%, because it was not possible to analyze each food item separately in the waste, rejections and adjustments, but such errors could not apply differentially in comparing the three diets. The final results of the analyses and computations are shown in table 5.

TABLE 5

Nutrients eaten,[1] grams per man per day, experiment AD with diets HCO, M and B

Fatty acids as glycerides	Diet HCO	Diet M	Diet B
Total saturated	34.1	35.4	74.8
Monoene			
cis	31.5	67.3	44.3
trans	34.0	0	0
Total monoene	65.5	67.3	44.3
Diene			
Nonconjugated			
cis, cis	14.0	24.1	5.8
cis trans	1.9	0	0
trans, trans	5.2	0	0
Conjugated			
cis, trans	1.6	0	0
trans, trans	2.9	0	0
Total diene	25.6	24.1	5.8
Triene	0.9	1.6	2.7
Total polyene	26.5	25.7	8.5
Total fat	126.1	128.4	127.6
Protein	77.1	76.5	76.9
Calories	2780	2742	2792
Supplement fat eaten	94.1	96.3	95.4

[1] Based on chemical analysis.

TABLE 6

Serum cholesterol values (mg per 100 ml) in experiment K when 30 gm of safflower oil were replaced by 30 gm of selectively hydrogenated safflower oil in the daily diet (27 men)

	Item	Serum cholesterol	
		Mean	SE
1	Safflower oil diet	196	±6.4
2	Hydrogenated safflower oil diet	206	±6.0
3	Hydrogenated safflower oil *minus* safflower oil	10	±2.4

RESULTS

The result of experiment K, summarized in table 6, shows that 30 gm of hydrogenated safflower oil in the daily diet produces a significantly ($P < 0.001$) higher serum cholesterol value than an equal amount of the natural oil. The result, indeed, corresponds closely with prediction with the equation (Keys et al., '59):

$$\triangle \text{ Chol.} = 2.68 \triangle \text{ S} - 1.23 \triangle \text{ P},$$

in which $\triangle$ S and $\triangle$ P are the differences between two diets in saturated and polyunsaturated fatty acid glycerides, measured as percentages of total diet calories. In the case of experiment K, the prediction is $\triangle$ cholesterol = 11 mg cholesterol per 100 ml of serum.

In experiment K no difference could be detected between the effect of the unnatural isomers in the hydrogenated safflower oil and that expected from the natural isomers used in the diets from which the equation was derived. Only small amounts of the unnatural isomers were involved, however.

The results of experiment N are given in table 7. With both safflower and corn oil the serum cholesterol level was considerably higher ($P < 0.001$) when the hydrogenated product was used in the diet. Again, this general result was predicted but it may be asked whether the magnitude of the dif-

TABLE 7

Serum cholesterol values (mg per 100 ml) in experiment N when 100 gm of natural oils were replaced by 100 gm of selectively hydrogenated oils

	Diet	No. of men	Serum cholesterol	
			Mean	SE
1	Safflower oil	12	160	±6.2
2	Hydrogenated safflower oil	12	185	±6.2
3	Corn oil	13	163	±9.3
4	Hydrogenated corn oil	13	184	±10.2
	Differences:			
5	Hydrogenated safflower *minus* safflower	12	25	±4.4
6	Hydrogenated corn *minus* corn	13	21	±3.8

ferences corresponds to expectation. Computation with the prediction equation indicates that somewhat larger differences in serum cholesterol level might have been expected from subsistence using diets having the same proportions of S and P fatty acids as in experiment N but with no unnatural isomers present. But that expectation involves the assumption that all other factors, including the responsiveness of the subjects to fats in the diet, were identical with those in the original experiments from which the prediction equation was derived. Clearly, no final conclusion about the effects of the unnatural isomers in the diet is justifiable from such an indirect estimate of expected differences.

Experiment AD, however, provides direct comparisons that are not subject to such uncertainties and assumptions. In comparison with diet M, diet HCO was strictly comparable except that a substantial proportion of the unsaturated fatty acids in the diet consisted of the unnatural *trans* and conjugated isomers. The effects on the lipids in the serum are shown in table 8.

The unnatural isomers in diet HCO in experiment AD were associated with highly significant elevations in the serum lipids as compared with the values in the same men subsisting on diet M. As expected, the butterfat diet (B) produced the highest values for cholesterol and phospholipid. The triglyceride levels with diet B were not as high as those with diet HCO, however. The unnatural fatty acid isomers in diet HCO had not only a definite effect in raising the serum cholesterol level; they also produced remarkably high concentrations of triglycerides in the serum.

DISCUSSION

Experiments K and N demonstrated that hydrogenation of unsaturated food oils produces higher serum cholesterol values when these materials are fed in the human diet. The design of those experiments, however, did not make it possible to decide whether a specific effect of the unnatural fatty acid isomers contributed to the result. Experiment AD clearly showed that the net effect of the 46 gm of unnatural isomers was to raise the serum cholesterol level considerably above that produced by a diet equivalent in amounts of saturated, monoene and diene fatty acids as natural isomers. Moreover, the diet containing these unnatural isomers caused an increase in the phospholipids and a very large increase in the triglycerides in the serum.

It is not possible to estimate the contribution to these effects in the blood serum of each of the various unnatural isomers in diet HCO. *Trans* monoene accounts for three-quarters of the unnatural isomers in diet HCO; hence it may be that it also accounts for most of the special effect on the serum lipids. If we simply speculate that the small amounts of *trans* and conjugated dienes in diet HCO had little or no effect,

TABLE 8

Serum lipids (mg per 100 ml) in experiment AD, mean values for 23 men

	Diet	Total cholesterol		Total phospholipids		Triglycerides	
		Mean	SE	Mean	SE	Mean	SE
1	Diet M	188	±6.8	208	±5.9	129	±10.7
2	Diet HCO	209	±9.0	226	±8.5	190	±25.4
3	Diet B	233	±9.1	245	±7.4	146	±14.4
	Differences:						
4	Diet HCO *minus* diet M	+21	±3.9	+18	±4.8	+61	±20.4
		$P < 0.0001$		$P = 0.004$		$P = 0.007$	
5	Diet B *minus* diet HCO	+24	±4.4	+19	±5.4	−44	±23.0
		$P < 0.0001$		$P = 0.002$		$P = 0.07$	
6	Diet B *minus* diet M	+45	±4.3	+37	±4.2	+17	±13.1
		$P < 0.0001$		$P < 0.0001$		$P = 0.2$	

then it is possible to estimate the specific effect of the *trans* monoene which provided 11% of the total calories in diet HCO. This difference from diet M was associated with a cholesterol difference of 24 mg per 100 ml of serum. The suggestion is that, whereas the *cis* monoene oleic acid has no serum cholesterol effect in man (Keys et al., '58), *trans* monoene has an effect close to the average of that produced by the saturated fatty acids with chain lengths from 12 C to 18 C. As indicated in the prediction equation, these saturated fatty acids have an effect indicated by the coefficient + 2.68; the corresponding coefficient for the mixed *trans* monoenes would be 24/11 = + 2.1. But final answers about the effects of the various unnatural isomers must await special experiments in which the several isomers are isolated variables.

Detailed final answers to this question will be extremely difficult to obtain. Hydrogenation of linoleic acid can produce at least 18 isomeric monoenes besides a number of geometrical and positional isomeric dienes (Allen and Johnston, '60). At least some of the *trans* isomers can be metabolized (Melnick and Deuel, '54; Allen and Johnston, '60), and Mattson ('60) has shown that the geometrical isomers of linoleic acid that are devoid of essential fatty acid activity do not interfere with the utilization of *cis, cis* linoleic acid. These facts suggest that the effect of these fatty acids on the serum cholesterol level may not be related to differences in the chemistry of intermediary metabolism but may be dependent on the way in which the fatty acids are digested, absorbed and transported in the blood.

Since hydrogenated fats are an important part of the diet in many parts of the world, this effect on serum lipids is of practical importance. The proportion of *trans* isomers in commercial hydrogenated fats seems to be lower than that in the hydrogenated fats used in this experiment (Sreenivasan and Brown, '56). Consequently, it is likely that such commercial preparations will produce lower lipid levels than those reported here.

SUMMARY

Selectively hydrogenated oils were exchanged for the corresponding natural oils in the diet of physically healthy men in a mental hospital. In 27 men a mean rise of 10 mg of cholesterol per 100 ml of serum resulted from the exchange of hydrogenated for natural safflower oil fed at the level of 30 gm in the daily diet. In 12 men similar exchange of those oils at the level of 100 gm daily produced a mean rise of 25 mg of cholesterol per 100 ml. In 13 men substitution of hydrogenated for natural corn oil fed at the level of 100 gm daily produced a mean rise in cholesterol of 21 mg per 100 ml.

In another experiment on 23 men the variable in the daily diet was 95 gm of fat, comparison being made between a mixture of natural vegetable oils and a hydrogenated product with the same proportions of saturated, monoene and diene fatty acids but containing 36% of *trans* monoene and 12% of *trans* or conjugated diene. The hydrogenated fat produced mean rises, per 100 ml of serum, of 21, 18, and 61 mg of cholesterol, phospholipids, and triglycerides, respectively.

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Amino Acid Imbalance: Rations Low in Tryptophan, Methionine or Lysine and the Efficiency of Utilization of Nitrogen in Imbalanced Rations[1]

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We have previously shown that an amino acid imbalance as defined by Harper ('59) can be induced in the growing chicken fed diets in which either lysine (Fisher et al., '60a) or arginine (Fisher et al., '60b) were limiting. These earlier studies suggested to us that the imbalance thus created was actually an exaggeration of an existing amino acid deficiency or the creation of a new one without evidence that the imposed imbalance had impaired the utilization of either total protein or that of the limiting amino acid. Previous experiments with rats and chickens (Sauberlich and Salmon, '55; Kumta and Harper, '60; Griminger, et al., '56) have suggested that inferior utilization of a growth-limiting amino acid resulted when protein or amino acid mixtures, complete except for the one limiting amino acid, were added to diets containing protein deficient in one or more amino acids. Recently Kumta and Harper ('61) showed that rats given insulin injections increased their food intake when supplied with an imbalanced ration and consequently also grew at a normal rate.

The present studies were undertaken (1) to extend the study of amino acid imbalance in the chicken from lysine and arginine to two other essential amino acids —tryptophan and methionine—and thereby to test the specificity of the condition; and (2) to compare the efficiency of utilization of the limiting amino acid and total protein under conditions of imbalance.

EXPERIMENTAL

Day-old crossbred chickens were used in the three trials to be reported. Until they were one week of age, they received a standard chick-starting ration, at which time they were assigned by weight to duplicate lots of 7, 5 and 10 birds, respectively, for experiments 1, 2 and 3. Basal rations and the composition of the amino acid mixture used to create an imbalance are shown in table 1. In the third experiment, diets containing three levels of energy were designed to insure equal food consumption of balanced and imbalanced rations. Low-energy rations were formulated by substituting 10% of fiber for glucose. The medium-energy rations were designed to be essentially isocaloric with rations previously used to produce and correct an imbalance (Fisher et al., '60), and supplementation with 6% of corn oil at the expense of glucose constituted the high-energy rations. All experimental rations were fed ad libitum for a two-week period, and body weights and feed consumption recorded at the end of the first and second week. In trial 3 the birds from certain lots (see table 2) were starved for 12 hours at the end of the experiment and were then killed with chloroform and dried to constant weight in a forced-air oven at 85°C. Moisture was determined as the difference between the final dry weight and the wet weight prior to killing. Nitrogen and fat were determined on pooled samples from each replicate after the dried carcasses had been finely ground and thoroughly mixed. Nitrogen was determined by semimicro Kjeldahl analysis and fat was determined by extraction with chloroform-methanol (2:1) and subsequent evaporation of solvent and weighing of the residue.

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TABLE 1

Composition of basal rations[1]

Ingredient	Rations (Amount)			Imbalancing amino acid mixture[2]	
	Trial 1	Trial 2	Trial 3	Amino acid	Amount
	%	%	%		% of mixture
Dextrin	50.00			DL-tryptophan	2.3
Starch	16.96			DL-methionine	2.1
Hydrolyzed casein	14.00			L-cystine	2.4
Sesame meal			24.00	DL-isoleucine	7.7
Peanut meal		18.20		DL-threonine	7.3
Isolated soy protein[3]		7.80		DL-valine	9.7
Alfalfa meal	3.00			L-arginine·HCl	7.3
Mineral mix[4]	4.94	4.94	4.94	DL-phenylalanine	6.1
Fiber[5]	2.00			L-tyrosine	3.0
Corn oil	3.00	3.00	2.00	L-glutamic acid	21.3
B-vitamin mix[6]	0.15	0.15	0.15	L-histidine HCl·H_2O	3.5
Vitamin A, D and E mix[6]	0.10	0.10	0.10	L-lysine·HCl	9.2
Choline chloride (70% conc.)	0.30	0.30	0.30	L-leucine	5.8
L-Arginine·HCl	0.60			glycine	12.1
L-Lysine·HCl		0.40			
DL-Methionine	0.15				
Glycine	0.60				
Glucose	to 100	to 100	to 100		

[1] The protein content of the three rations varied between 11 and 13% (N × 6.25).
[2] In trials 1, 2 and 3, respectively, tryptophan, methionine and lysine were omitted from the mixture. In all experiments the amino acid mixture was added at the expense of glucose.
[3] ADM Assay Protein C-1, Archer-Daniels-Midland Company, Cincinnati.
[4] For composition see Fisher et al., ('60a).
[5] Solka Floc, Brown Company, Berlin, New Hampshire.
[6] For composition see Fisher and Johnson ('56).

RESULTS

Trial 1. In this experiment a tryptophan-free hydrolyzed casein ration was used to study amino acid imbalance in relation to tryptophan. Four treatments were compared: an adequate and an inadequate tryptophan level, both in the presence and absence of the imbalancing amino acid mixture. As shown in figure 1 the addition of the tryptophan-free, but otherwise balanced, amino acid mixture to the diet containing a suboptimal level of tryptophan (0.7% of the protein) caused a depression both in food consumption and growth. The addition of more tryptophan to the imbalanced ration not only overcame the depression but improved the growth rate beyond that with the rations not supplemented with the imbalancing amino acid mixture. This, of course, was to be expected since a relatively low protein diet (12%) was fed and the chicken could therefore utilize the amino acid mixture for growth beyond that permitted by the low-protein level when adequate tryptophan was also provided.

Trial 2. In this experiment methionine was studied as the limiting amino acid, using a peanut meal-isolated soybean protein[2] combination. A growth depression of a similar order of magnitude as shown for tryptophan, and as previously observed with lysine and arginine (Fisher et al., '60a; '60b), was also induced when an amino acid mixture deficient in methionine but otherwise adequate was added to a methionine-low ration (fig. 2). In conformity with previous experience with low-protein diets, growth was again markedly improved when adequate methionine was provided in the imbalanced ration.

Trial 3. This experiment was designed to gain more direct information on the efficiency with which the nitrogen portion of an imbalanced ration is utilized for growth. In this study lysine was the limiting amino acid and sesame protein

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

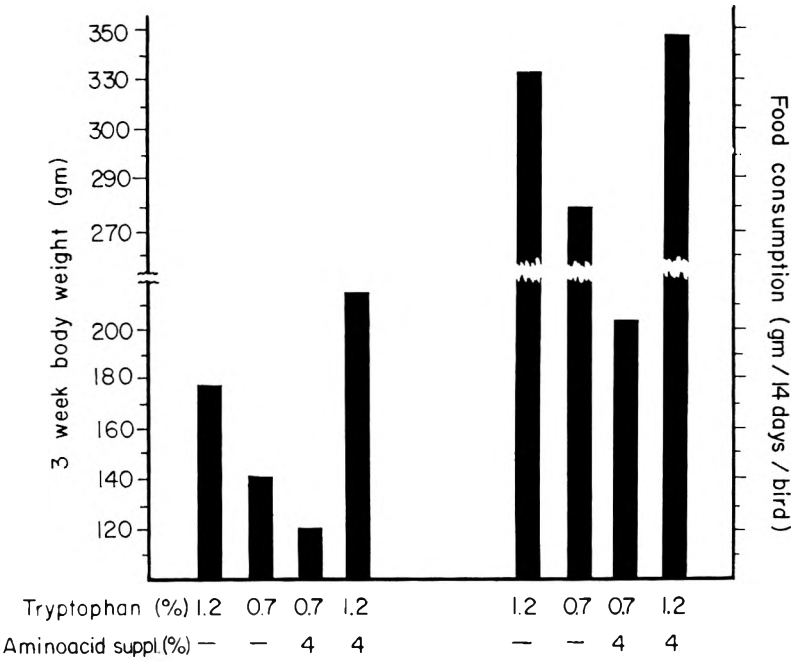

Fig. 1 Food consumption and body weights of chickens fed a ration balanced or imbalanced with respect to tryptophan. Bars on left hand of figure refer to body weights, those on the right to food consumption with the divisions on the right ordinate corresponding to those on the left ordinate. Tryptophan expressed as percentage of protein, amino acid mix added as percentage of diet.

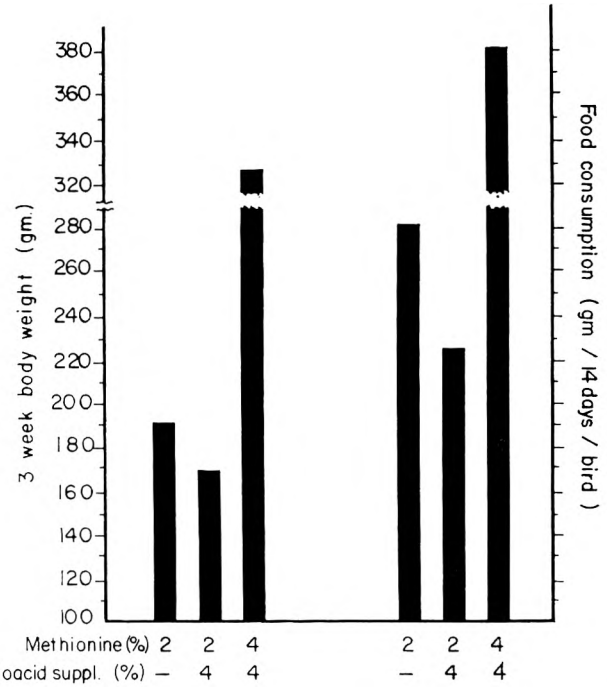

Fig. 2 Food consumption and body weights of chickens fed a ration balanced or imbalanced with respect to methionine. Bars on left hand of figure refer to body weights, those on the right to food consumption with the divisions on the right ordinate corresponding to those on the left ordinate. Methionine expressed as percentage of protein, amino acid mix added as percentage of diet.

was used as in earlier studies (Fisher et al., '60a).

At each energy level lysine deficiency, amino acid imbalance in relation to lysine, and correction of the imbalance were studied. Food consumption and growth data for the lysine imbalance are shown in figures 3 and 4. At the low- and medium-energy levels the growth depression and decrease in food intake due to the imbalancing amino acid mixture were typical and similar to those for tryptophan

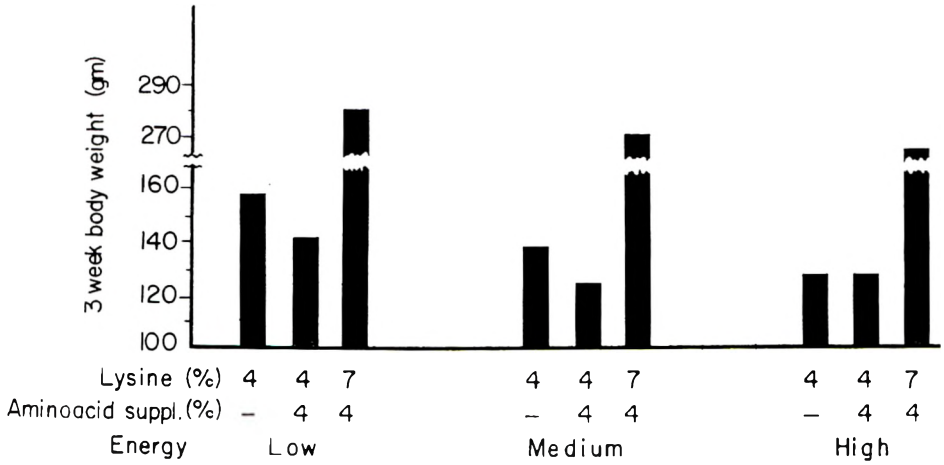

Fig. 3 Body weights of chickens given rations balanced or imbalanced with respect to lysine and with differing dietary energy levels. Lysine expressed as percentage of protein, amino acid mix added as percentage of diet.

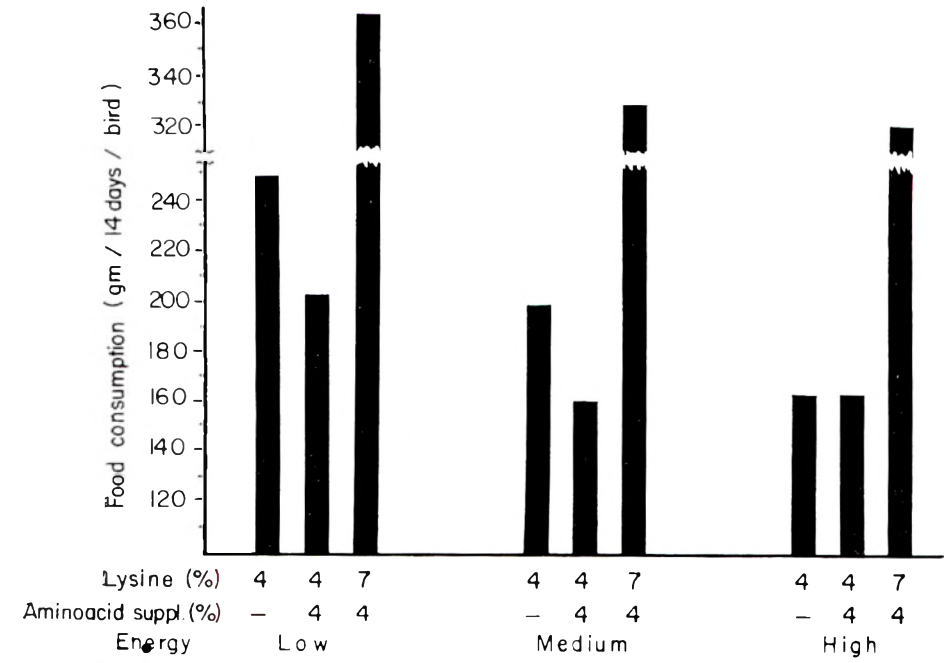

Fig. 4 Food consumption of chickens given rations balanced or imbalanced with respect to lysine and with differing dietary energy levels. Lysine expressed as percentage of protein, amino acid mix added as percentage of diet.

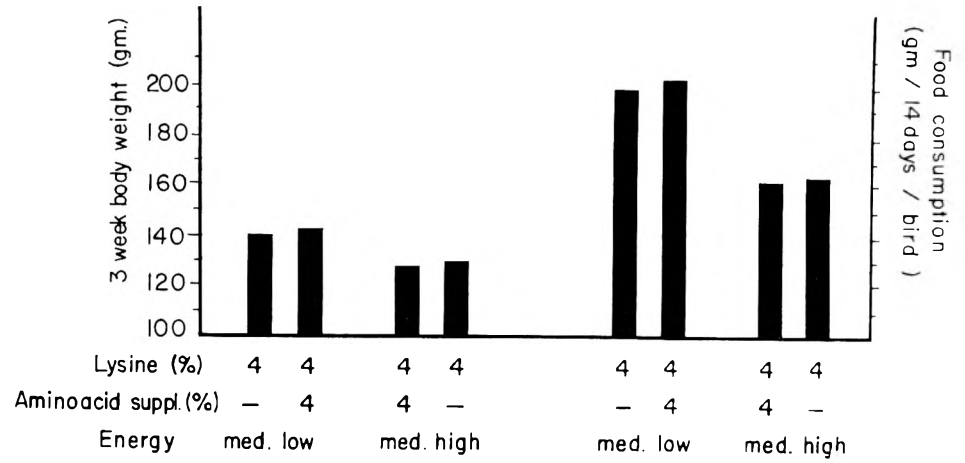

Fig. 5 Comparison of body weight and food consumption of chickens fed balanced or imbalanced rations with different dietary energy levels. Lysine expressed as percentage of protein, amino acid mix added as percentage of diet.

and methionine. At the high-energy level, however, there was neither a growth depression nor decreased food consumption. This was not unexpected and will be discussed later. In figure 5 are shown the comparisons for which this experiment was designed. The imbalanced group receiving the low-energy diet consumed essentially the same amount of food and therefore of lysine and grew to the same extent as did the balanced group (not receiving the amino acid supplement) fed the medium-energy ration. Similarly, the high-energy group without the addition of the imbalancing amino acid mixture consumed the same quantity of food and therefore of lysine, and grew at the same rate as did the imbalanced group fed the medium-energy level. These gross observations suggest that there is no loss in efficiency with which the protein and limiting amino acid portion of an imbalanced ration are utilized. The values in table 2 confirm this suggestion by showing that the carcass nitrogen content of the imbalanced groups is actually higher than that of the corresponding balanced groups which consumed the same amount of feed and grew to the same body weight. The higher carcass nitrogen content is no doubt due to the lower energy intake with the imbalanced rations, a fact which is also reflected in a lower carcass fat content.

TABLE 2

Carcass composition of birds fed balanced or imbalanced rations (trial 3)[1]

Dietary variables[2]		Carcass composition		
Energy	Imbalancing amino acid mixture	Moisture	Fat	Nitrogen
	%	%	*% dry weight*	*% dry weight*
Medium	–	68.8	35.0	8.70
Low	4	69.8	31.7	9.66
High	–	68.5	36.6	8.40
Medium	4	68.8	31.9	9.29

[1] Determinations were carried out on duplicate or triplicate samples of the pooled carcasses of duplicate groups/treatment.

[2] Lysine content of ration was suboptimal at 4% of the protein level.

DISCUSSION

The results presented extend the concept of amino acid imbalance (as defined by Harper, '59) to two additional amino acids. The induction of a depression in food intake and in growth by supplementation with an amino acid mixture deficient in one essential amino acid that is also limiting in the original ration has now been demonstrated in the growing chicken for the amino acids lysine, arginine, tryptophan, and methionine, thus suggesting that the phenomenon is of a general nature.

The efficiency comparisons of trial 3 clearly confirm our previous suggestion (Fisher et al., '60a) that there is no apparent impairment in the utilization of protein or of the limiting amino acid in an imbalanced ration. Thus, regardless of the addition of the imbalancing amino acid mixture, a direct relationship was observed between feed and therefore lysine consumption and growth rate (fig. 5). It is well known that an increase in caloric intake increases nitrogen retention (Munro and Naismith, '53) particularly at suboptimal levels of energy. Hence, despite a lower energy intake chicks receiving the imbalanced diets still utilized protein and the limiting amino acid (lysine) to the same extent as chicks consuming a higher level of energy in the absence of the imbalancing amino acid mixture (table 2). The lack of impairment in utilization suggests that the conditions described by the term *imbalance*, as used in its present context, cannot be differentiated from an amino acid *deficiency*. This becomes more apparent when one expresses amino acid composition or requirement as a percentage of the dietary protein level rather than as a percentage of the diet. Such expression reveals that the addition of an imbalanced amino acid mixture dilutes the most limiting amino acid to the point of exaggerating an existing deficiency. Since the growth depression cannot be explained in terms of impaired food utilization, it can be related only to the reduced food intake. Such a reduction is also observed in amino acid as well as other nutrient deficiencies.

The lack of a growth depression with the high-energy ration that contained the imbalancing amino acid mixture in trial 3 confirms an earlier observation that an imbalance of this type will occur only when the ration permits a fair level of growth. Note that the widened ratio between calories and protein in the high energy rations reduced growth in comparison with the corresponding rations at the lower energy levels.

The exact mechanism of the depressed food intake continues to elude us. We have studied changes in plasma amino nitrogen in chickens fed imbalanced and normal rations. In two separate experiments an increased plasma amino nitrogen level was observed due to an imbalance. Addition of the most limiting amino acid reduced the amino nitrogen level to approximately that of the control group (table 3). These results are similar to the recent observations by Kumta and Harper ('61) on blood urea levels of growing rats given balanced and imbalanced rations.

TABLE 3

Plasma amino nitrogen levels of chickens fed rations balanced or imbalanced in respect to lysine

Dietary variables		Plasma amino nitrogen[1]	
Lysine	Amino acid mix	Exp. 1	Exp. 2
% of protein	*% of diet*	*mg/100 ml*	*mg/100 ml*
4		10.0 ± 0.4	12.8 ± 0.5
4	4	12.2 ± 0.5	15.5 ± 0.8
7	4	9.0 ± 0.2	13.5 ± 0.7[2]

[1] Mean value with its standard error for 10 chickens (exp. 1) and 14 chickens (exp. 2) Amino nitrogen was determined by the method of Danielson as presented in Hawk et al. ('54).

[2] Value represents only 5 chickens.

SUMMARY

Three experiments were conducted with growing chickens. In the first and second of these, an amino acid imbalance (deficiency) was created with rations low in tryptophan or methionine. In the third experiment diets containing three levels of energy were formulated in such a manner that birds consumed equal quantities of food and therefore of protein and of the most limiting amino acid (lysine) when fed rations that were either imbalanced or balanced. This permitted a comparison of the efficiency of utilization of the protein and of the most limiting amino acid. No impairment in utilization occurred in terms of either body weight gain or carcass nitrogen content of birds fed imbalanced versus normal rations. The results indicate that the term imbalance as used in its present context cannot be differentiated from an amino acid deficiency.

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Long- and Short-Term Studies with Free Amino Acid Diets for the Laying Hen[1,2]

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Work has been continued in an attempt to formulate a satisfactory diet for the laying hen in which the nitrogen is supplied solely by crystalline amino acids. Earlier workers (Grau and Taylor, '48 and Ingram et al., '50) observed that purified diets containing amino acids in place of intact protein were unsatisfactory in that body weight was not maintained and egg production generally ceased during the first week. Fisher and Johnson ('56) reported that egg production could be maintained for 30 days by hens fed a free amino acid diet. The 4 birds used to demonstrate that egg production could be maintained beyond a two-week period averaged approximately 49% production. No information about body weight maintenance over the 30 days was presented.

Johnson and Fisher ('58) fed pullets for three weeks a free amino acid diet in which the essential amino acids were reduced to the minimal levels for egg production. A 10% increase in all of the essential amino acids increased egg production from 2.6 to 3.6 eggs per bird per week (calculated).

Adkins and co-workers ('59) demonstrated that egg production and body weight were not maintained satisfactorily by pullets fed a free amino acid diet. Four different amino acid mixtures were tried with various modifications. Fair production (52%) was maintained for 5 weeks by 4 pullets fed a diet containing an amino acid mixture consisting of 15 amino acids, but there was a large loss of body weight. The addition of 2% of intact protein to other amino acid diets extended egg production at a low level to 6 weeks and improved body weight maintenance.

In this report, short-term and long-term studies are described in which a protein-free, amino acid diet that supported satisfactory chick growth was tested with laying pullets, hens, and quail. To the knowledge of the authors, this is the first demonstration of body weight maintenance for 6 months by hens fed a diet in which all of the protein was replaced by free amino acids.

EXPERIMENTAL

Single Comb White Leghorn pullets or hens from the University flock were used in these studies. They were housed in individual laying batteries with raised-wire floors and were supplied feed, water, and oyster shell ad libitum. Each day all the eggs obtained were pedigree marked and weighed individually. Some eggs were broken out and the interior quality determined using the method of Haugh ('37). The birds were artificially inseminated with pooled semen collected from New Hampshire cockerels and approximately one-half of all the eggs were incubated. All of the incubated eggs that failed to hatch were broken out and examined to determine the age of death of the embryo and the occurrence of abnormalities. Several groups of the chicks that hatched were reared in an electrically heated, chick-starting battery. These chicks were fed either a practical ration for 4 weeks or an amino acid diet for two weeks. All chicks, pullets and hens were weighed weekly.

The pullets or hens were fed a purified diet (consisting of 20% of casein and 8% of gelatin supplemented with 0.1% of DL-methionine and the constant ingredients

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given in table 1) for two weeks prior to being fed the experimental diets to accustom them to a purified diet. Only pullets or hens averaging 60% production or higher during the pre-test period were used in the experiments. An attempt was made to assign them so that the rate of lay and the weights of groups would be fairly uniform. Once they started to be fed the experimental diet they were not taken off the diet until the termination of the experiment. The pullets were females about 8 months of age and the hens were about 20 months of age when experiment 2 began.

The crystalline amino acids were premixed in the manner previously used in this laboratory (Adkins et al., '58).

Corn starch that had been autoclaved for two or three hours at 15 pounds pressure, then dried and ground, was the carbohydrate used in all experiments.

TABLE 1

Constant ingredients of the basal diet

	%
Cellulose[1]	3.0
Salts V[3]	6.0
Antiacid[4]	1.0
$NaHCO_3$	1.0
Choline chloride (70% aqueous)	0.1
α-Tocopheryl acetate (0.6 mg/gm)	0.1
Ethoxyquin[5]	0.0125
	%
Vitamin mixture[2]	0.5
Feeding oil (800 IU vitamin A/300 ICU vitamin D_3)	0.1
Vitamin A acetate (25,000 IU/gm)	0.1
Vitamin D_3 (1500 IU/gm)	0.08
Crude soybean oil	10.0
Cooked starch (to 100%)	+

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] Mixture supplies the following vitamins in mg/kg of diet: thiamine·HCl, 6.0; biotin, 0.2; pyridoxine·HCl, 4.0; 2-methyl-1,4-naphthoquinone, 0.5; folic acid, 4.0 riboflavin, 6.0; D-calcium pantothenate, 20.0; niacin, 50.0; inositol, 1000; and vitamin B_{12}, 0.03.

[3] Briggs et al. ('43).

[4] Consists of three parts magnesium trisilicate and one part aluminum hydroxide.

[5] 1,2-dehydro-6-ethoxy, 2,2,4-trimethylquinoline, Santoquin, Monsanto Chemical Company, St. Louis.

RESULTS AND DISCUSSION

Experiment 1. The amino acid diet[3] used in our earlier work (diet D, tables 1 and 2) was fed to 6 pullets for 4 weeks. These pullets averaged only 20.9% production as compared with 82.2% for the two pullets fed a practical laying mash. The pullets fed the amino acid diet lost on the average 173 gm in body weight and the egg weight dropped 4.3 gm as compared with an average loss of only 12 gm in body weight and a decline of 1 gm in egg weight for the controls.

TABLE 2

Composition of amino acid mixture

Amino acid	Percentage of diet				
	D	E	F	G	H
L-Arginine·HCl	0.91	1.6	1.2	2.0	1.0
L-Glutamic acid	7.5	7.0	7.0	7.0	7.5
Glycine	1.5	2.0	2.0	2.0	1.5
L-Histidine·HCl	0.25	0.5	0.4	0.6	0.3
L-Lysine·HCl (95% L)	0.63	1.3	0.9	1.6	0.8
DL-Methionine	0.8	1.0	0.8	1.2	0.6
DL-Threonine	0.8	1.4	0.9	1.8	0.9
DL-Tryptophan	0.3	0.5	0.4	0.6	0.3
DL-Valine	1.0	1.9	1.6	2.2	1.1
DL-Isoleucine (50% L)	1.2	1.6	1.2	2.0	1.0
L-Leucine	0.7	1.7	1.4	2.0	1.0
DL-Phenylalanine	0.8	1.0	1.0	1.0	0.5
L-Tyrosine	0.4	0.9	0.9	0.9	0.45

The results of this experiment were very similar to those reported earlier by Adkins et al. ('59).

Experiment 2. Since an amino acid diet had been formulated in our laboratory that would support satisfactory chick growth (7 to 12 gm a day), this diet (diet G, table 2) was fed in the next experiment to both pullets and hens. Diet G contained the equivalent of 19.6% of crude protein (N × 6.25). A new diet (diet H, table 2) containing one-half the levels of the indispensable amino acids in the chick diet was also fed at this time. This diet contained 7.5% of L-glutamic acid and 1.5% of glycine.

The combined data for hens and pullets are given in table 3. Three out of 4 of the hens and pullets fed the amino acid diet

[3] The authors are indebted to Dow Chemical Company, Midland, Michigan, for kindly furnishing DL-valine, DL-phenylalanine, DL-leucine, D-allo L-isoleucine, DL-tryptophan, DL-threonine and DL-tyrosine; Merck and Company, Inc., Rahway, New Jersey, for L-lysine·HCl; E. I. du Pont de Nemours Company, Wilmington, Delaware, for DL-methionine; and the Monsanto Chemical Company, St. Louis, for DL-phenylalanine, DL-valine and DL-isoleucine used in these experiments.

G (table 2) laid at a good rate for the 6-week experimental period. These three averaged 66.7% production (range of 62 to 74%) as compared with 67.8% for the hens and pullets fed the casein-gelatin control diet and 61.9% for the birds fed the practical control diet. One pullet in the group fed this amino acid diet, however, laid only 13 eggs (31% production) during the period. Diets D and H did not support satisfactory egg production although the birds continued to lay at a low rate.

Pullets fed diet G were able to maintain body weight, whereas hens lost weight. On the average, this group showed a gain of 20 gm at the end of 6 weeks. There was no change in the average weight of eggs from the fowl fed this diet. Feed conversion was comparable to that obtained with the control diets. There was a decline in both egg weight and body weight of birds fed diets D and H. Those hens or pullets that did show a gain in body weight in these groups tended to be poor producers.

Hatchability and chick growth. Hatchability of fertile eggs was excellent for all groups throughout all of the experiments.

Chicks from dams fed the amino acid diets were on the average 2.4 gm lighter at the time of hatching than chicks from control birds (table 4). This figure represents the average weight difference of chicks from dams fed diets D, G, and H. Most of these differences were caused by smaller chicks fed diets D and H. The greatest difference in weight at hatching was between the two groups of chicks from hens as compared with those from pullets. The chicks from pullets fed the amino acid diets averaged only 0.4 gm less than those from the control group.

After being fed a practical-type chick starting diet for 4 weeks, chicks from hens fed the amino acid diets were, on the average, 23.6 gm heavier than those from the control hens (table 4). When day-old chicks from pullets fed an amino acid diet were supplied with an amino acid diet, contain-

TABLE 3

Performance of laying birds fed free amino acid diets (exp. 2)

Group[1] no.	Diet fed	Egg production: Eggs/group/week 0	1	2	3	4	5	6	Average	Av. change in body weight	Av. change in egg weight	Av. feed conversion
									%	*gm/bird*	*gm*	*gm feed/egg*
1	Casein gelatin control	18	20	20	19	18	17	20	67.8	68.7	5.1	151
2	Practical control	20	17	16	17	19	17	18	61.9	25.0	1.8	187
3	Amino acid G (table 2)	19	15	16	15	15	18	18	57.7	20.0	0.0	162
4	Amino acid D (table 2)	19	13	12	9	8	9	5	33.3	−40.0	−2.1	261
5	Amino acid H (table 2)	18	15	7	10	8	10	7	33.9	−110.0	−2.7	383

[1] Four birds/group: 2 S.C.W.L. hens in their second laying year and 2 S.C.W.L. pullets.

TABLE 4

Growth rate of chicks fed a practical chick starting ration to 4 weeks of age

Diet fed dam	Average starting weight	Hatch: 1	2	3	4	5	Av.[1]
		Average gain					
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Control[2] (hen)	39.7	290.5	243.0	—	205.7	202.8	224.6
Amino acid[3] (hen)	34.5	322.1	269.9	284.3	227.1	208.8	242.3
Control (pullet)	31.7	231.7	242.2	245.2	195.3	159.5	194.1
Amino acid (pullet)	32.1	263.9	221.1	244.7	197.5	215.6	223.7

[1] Total of 150 chicks.
[2] Chicks combined from birds fed casein-gelatin control diet and practical control diet.
[3] Chicks combined from birds fed amino acid diets D, G, and H, table 2.

ing mixture G, table 2, the growth rate of these chicks during the second week fell below that of chicks hatched from pullets fed a practical ration (table 5). The lag observed during the second week in this experiment and later in other experiments may be related to the absence of unidentified chick growth factors from the amino acid diet.

TABLE 5

Growth of chicks hatched from pullets fed an amino acid diet (exp. 2)

Diet fed dam	Diet fed chicks[1]	Av. gain weeks		
		1	2	Total
		gm	*gm*	*gm*
Practical control	Casein[2] control	18.8	36.4	55.2
Amino acid[3]	Casein control	21.4	39.4	60.8
Practical control	Amino acid G[4] (table 2)	22.4	29.0	51.4
Amino acid	Amino acid G (table 2)	22.8	20.2	43.0

[1] Five N.H. × S.C.W.L. day-old chicks fed diet indicated.
[2] 20% crude casein.
[3] Pullets were fed diet D, H or G, table 2.
[4] Additional amounts of vitamins added.

TABLE 6

Internal quality of eggs laid by birds fed a free amino acid diet (exp. 2)

Diet fed	Average Haugh[1] units		
	Hens	Pullets	Average
Practical control	82 (28)[2]	86 (48)	84 (76)
Amino acid G (table 2)	84 (34)	88 (42)	86 (76)

[1] Haugh, R. R. ('37).
[2] Figures in parentheses indicate number of eggs broken-out.

Egg quality. Except for the difference in yolk color, due to the low level of pigment in the diet, eggs from birds fed the free amino acid diet G were as high in internal quality as those laid by birds fed a practical ration. Haugh Unit values are given in table 6. A Haugh Unit is a measure of the condition of the albumen as judged by its height and the weight of the egg. The height was measured midway between the edge of the yolk and the outer edge of the thick white with a tripod micrometer.

Egg production. Since diet G apparently contained a mixture of amino acids that would support satisfactory production and weight maintenance, birds fed this diet were continued on experiment for 6 months. Individual egg production records beyond 6 weeks are shown in table 7. One hen (no. 20) began molting the 8th week and remained out of production for 5 weeks. She began laying again while completing the molt. This bird replaced both body and wing feathers normally. Hen number 11, fed a practical laying ration, also went through a complete molt during this period, 9th week to the 20th week, but managed to maintain a low rate of production throughout. The improved egg production and body weight maintenance of birds fed amino acid diet G are due probably to the increased levels of the indispen-

TABLE 7

Egg production of birds fed amino acid diets beyond 6 weeks (exp. 2)

Group no. (as in table 3)	Bird no.	Weeks																		Total eggs in 24 weeks	Gain 0–24 weeks
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
																					gm
2[1]	11 H[2]	4	4	1	2	2	4	0	1	4	2	3	1	2	0	2	2	2	2	60	265
	12 H	4	4	5	5	4	5	4	5	2	0	4	5	5	5	4	4	4	4	99	130
	41 P	5	4	6	6	4	4	4	3	0	4	5	4	5	6	4	5	5	4	107	300
	42 P	7	4	6	7	5	5	6	5	6	5	5	5	6	6	5	5	4	4	123	490
3[3]	19 H	5	3	0	2	4	2	5	4	4	4	5	7	5	4[4]	2	4	4	3	94	100
	20 H	3	M	0	0	0	0	1	4	0	3	4	4	4	3[4]	2	1	3	2	60	300
	37 P	5	5	5	5	3	2[4]	4	4	5	3	4	0	4	4	4	4	4	2	98	180
	38 P	4	4	4	2	3	2[5]	5	3	1	3	4	2	4	3[4]	1	2	4	2	66	90

[1] Fed a practical laying ration.
[2] H denotes hen, a female about 20 months of age at start of experiment that had been through one laying year; P denotes pullet, a female about 8 months of age at the start of the experiment in her first laying year.
[3] Fed free amino acid diet G.
[4] Fed diet F, table 2.
[5] Fed diet E, table 2.

sable amino acids in this diet. Increasing the total nitrogen level of diet D by the addition of L-glutamic acid and glycine did not improve the egg production of one pullet.

At the end of 12 weeks the pullets fed the amino acid diet G were supplied with a diet in which the levels of some amino acids had been reduced by 20 to 40% (diets E and F, table 2). The hens were also switched to diet F containing lower levels of amino acids except for glutamic acid, phenylalanine, tyrosine, and glycine at the end of 20 weeks. Egg production of birds fed these diets with lower amino acid levels (diets E and F, table 2) dropped approximately 8.5%. The production of pullet no. 38 was not lowered, however, during the 4 weeks this bird was fed diet E. Over the entire 24 weeks the birds fed the free amino acid diets averaged 47.2% production as compared with 57.9% for the birds fed a practical laying ration. During portions of this period, the birds were fed amino acid mixtures that were much lower in certain amino acids than diet G. This may account for some of the drop in percentage production. Body weight was maintained but there was a drop in egg weight.

Experiment 3. This experiment was conducted to determine whether a diet containing lower levels of amino acids would maintain egg production of pullets not previously fed free amino acids. Two pullets were fed a diet in which all of the amino acids in diet G, table 2 were reduced by 40%, except for L-glutamic acid which was kept at 7% of the diet. Two pullets were given this diet supplemented with 0.3% of DL-methionine and 0.3% of DL-threonine. The results of this experiment are given in table 8. One bird (no. 6) did not consume much feed initially and started molting during the first week of the experiment, laying only two eggs for the 14 days. This bird laid on the 15th day. Excluding this one, egg production was as good with the diet containing 60% of the amino acids in diet G as it was on diet G. Body weight was maintained with the amino acid diet by all but one bird (no. 8) that lost 15 gm. The results of this experiment may be compared with the two-week results given in table 3. Birds fed diets D and H, table 2, in experiment 2 laid at a reduced rate during the second week, and lost weight the first week. As previously observed, feeding the amino acid diet resulted in a decline in egg weight by the second week. In this short-term study there did not appear to be any beneficial effect of the additional methionine and threonine.

The diet with the levels of the amino acids in diet G (table 2) reduced by 40%

TABLE 8

Performance of laying pullets fed reduced levels of amino acids in a free amino acid diet (exp. 3)

Bird no.	Diet fed	Egg production				Change in body weight	Change in egg weight	Total consumption
		0	1	2	Total			
		eggs/bird/week				*gm*	*gm*	*gm*
1	Practical control	4	5	5	10	25	1.8	2810
2	Practical	4	6	4	10	35	0.0	
3	G (table 2)	5	3	2	5	115	−3.3	2700
4	G (table 2)	5	4	6	10	45	−2.0	
5	60% of G	5	5	4	9	20	−0.3	2325
6	As no. 5	5	2[1]	0[1]	2	155	—	
7	As no. 5 + amino acids[2]	5	4	3	7	160	−1.0	2750
8	As no. 7	5	6	4	10	−15	−0.2	

[1] Pullet molted rapidly during the first 12 days of the experiment but came back into production on the 15th day.

[2] 0.3% DL-methionine and 0.3 DL-threonine added.

was excellent for body weight maintenance but did not support optimal egg production. It appears that a diet containing higher levels of the amino acids more closely meets the total amino acid needs of the laying hen. In table 9 are shown the theoretical amino acid requirements for the laying hen reported by Fisher ('61) and the actual intake expressed in milligrams per day per hen for a hen consuming daily approximately 98 gm of diet E, table 2.

TABLE 9

Amino acid requirement of the laying hen

Amino acid	Theoretical requirement[1]		Actual consumption[2]
	mg/day/ 1.5 kg hen		*mg/day/ 1.8 kg hen*
	A	B	
Cystine	191	153	—
Methionine	259	221	980
Histidine	139	170	397
Isoleucine	508	468	784
Leucine	508	638	1666
Lysine	443	468	970
Phenylalanine	376	391	490
Tyrosine	—	280	882
Threonine	500	360	686
Tryptophan	123	110	245
Valine	520	502	931
Arginine	581	680	1224

[1] Fisher, H., Feeds Illustrated, *12*: 8, 1961. Column A is based on the "theoretical considerations arising from a summation of the maintenance requirement plus the amino acids in a 50-gm egg." Column B gives "minimal requirements based on the whole-egg protein ratio technique."

[2] Actual amino acid intake of hen consuming daily approximately 98 gm of amino acid diet E, table 2. (D- forms are excluded except for DL-methionine.)

The actual consumption values are in every case above the theoretical values of Fisher ('61). It is recognized that diet E, table 2, probably contains an excess of amino acids for the hen but it was necessary to supply approximately these amounts of amino acids for optimal egg production. Diet F, table 2 which appears marginal for optimal egg production when consumed at a rate of 98 gm per day, also supplies higher quantities of amino acids than the suggested requirements based on theoretical calculations. Some D- forms of amino acids have been used, but except for methionine, they have been excluded from the calculations.

Experiment 4. Six quail (Corturnix Japanica) were housed in individual wire cages. Diet G or 20% casein (table 2), water and chick-size oyster shell were supplied ad libitum. Results of this preliminary trial are given in table 10. Only one quail fed the free amino acid diet was laying at a high rate during the 4th week. The average egg production for the entire group of birds fed the amino acid diet was 50.0% (range 25 to 78.5%) as compared with 80.9% for the birds fed a 20% crude casein diet. Quail fed this control diet gained on the average 1.3 gm and the quail fed the amino acid diet lost on the average 5.0 gm during the 4-week period. The poor performance of bird no. 4 is felt to be a reflection of a low feed intake. These results suggest that even amino acid diet G, which had given good results for the hen, was not very satisfactory for the Japanese quail.

TABLE 10

Performance of laying quail (Corturnix Japanica) fed a free amino acid diet (exp. 4)

Bird no.	Diet fed	Egg production						Change in body weight
		0	1	2	3	4	Total	
		eggs/bird/week						
1	20% Casein	3	7	7	5	6	25	3.0
2	20% Casein	6	5	6	5	3	19	14.0
3	20% Casein	4	7	6	6	5	24	−13.0
4	Amino acid G (table 2)	5	5	0	2	0	7	−15.0
5	Amino acid G (table 2)	5	3	6	6	7	22	−3.0
6	Amino acid G (table 2)	2	3	4	3	3	13	3.0

The quail were fed a practical laying ration containing 15% of protein prior to being fed the 20% casein diet. Egg production of quail with the casein diet was higher than that of those fed the practical diet. This may indicate that the protein requirement of the Japanese quail is greater than 15%.

SUMMARY

A protein-free, amino acid diet, equivalent in amino acid content to a diet containing 19.6% of crude protein, supported reasonably satisfactory egg production and body weight maintenance of hens and pullets for 6 months. To the knowledge of the authors, this is the first time laying birds have been maintained with a diet completely devoid of protein for this length of time. Since one hen completely molted and refeathered during the experiment, it appears that feather replacement progresses normally when an amino acid diet is fed.

Reducing the level of amino acids in this diet by 50% resulted in unsatisfactory performance. A 40% reduction of all amino acids, except L-glutamic acid, glycine, phenylalanine and tyrosine, resulted in a decrease in egg production by the birds previously fed higher levels of amino acids for 12 to 20 weeks.

Hatchability was not affected by feeding free amino acid diets. Growth of chicks from hens fed amino acid diets was comparable with that of chicks hatched from control hens when both groups were fed a practical diet. Interior quality of eggs from birds fed the amino acid diets measured by the method of Haugh was as high as that of eggs from control hens.

In a preliminary study, only one out of three Japanese quail had a good rate of egg production (78.5%) when fed an amino acid diet for 4 weeks. The data suggest that the protein requirement of the Japanese quail is greater than 15%.

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Influence of Dietary Fat on the Fatty Acid Composition of Monkey Erythrocytes[1]

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It has been known for many years that dietary fat influences the composition of depot fat in a variety of species (Deuel, '55). More recently there has been considerable interest in the influence of ingested fat on the fatty acid composition of structural lipids (Horwitt et al., '61). Horwitt et al. ('59) were able to show that alterations in dietary fat result in changes in the fatty acid composition of human erythrocytes and chick cerebella and it is known that the fatty acid composition of various tissues of the rat are affected by changes in dietary fat (Holman, '60; Witting et al., '61). Recent discussions of the syndrome of essential fatty acid deficiency in the rat and other animals are available (Holman, '60; Aaes-Jørgensen, '61).

With evidence accumulating concerning the role of lipids in active transport across membranes (Hokin and Hokin, '61) and in the electron transport phenomena (Green and Hatefi, '61), as well as a report by Collins ('60) which suggests that the metabolic activity of phospholipids is related to their fatty acid composition, knowledge of the influence of dietary fat on the lipid composition of cells becomes more important. The present report shows that dietary fat greatly influences the lipid composition of the erythrocytes of monkeys and a description of the syndrome of prolonged fat deficiency in the monkey is given.

EXPERIMENTAL

Pre-adolescent rhesus monkeys initially weighing between 1.5 and 2.5 kg were supplied with two diets, one containing very little fat and the other containing 8% of lard and 3% of cod liver oil. The latter diet was the basal diet previously described (Dinning and Day, '57) except that vitamin B_{12} was added to supply 0.05 to 0.1 μg per day per monkey and molecularly distilled lard[3] containing 2 to 3 μg of tocopherols per gm was used. The cod liver oil contained 180 μg of α-tocopherol per gm and contained (in per cent) palmitic acid, 13.2; palmitoleic acid, 7.6; oleic acid, 26.8; linoleic acid, 1.4; eicosamonoenoic acid, 10.4; eicosotetraenoic acid, 7.2; eicosapentaenoic acid, 7.6; and docosahexaenoic acid, 12 as the principal fatty acids. The low-fat diet was the same except that lard and cod liver oil were replaced by a similar weight of corn starch. It is estimated on the basis of prior experience[4] that the starch contained approximately 0.8 μg of tocopherol and 1.8 mg of linoleic acid per gm. The animals that were fed the low-fat diet were given 400 IU of vitamin D_3 and 2 mg of vitamin A acetate per day. Six monkeys received the diet containing lard and cod liver oil and 4 monkeys received the low-fat diet for approximately 550 days. Four of the monkeys receiving the lard and cod liver oil-containing diet and three of the monkeys receiving the low-fat diet were not given tocopherol supplement, while the

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[3] "Stripped lard" supplied through the courtesy of Dr. Stanley Ames, Distillation Products, Inc., Rochester, New York.

[4] Unpublished experiments. Witting, L. A. and G. J. Wright.

others were given 240 mg of DL-α-tocopherol per week.

The absence of a vitamin E supplement did not influence the appearance or growth of the animals or the lipid composition of the erythrocytes during the time period covered by this report. Vitamin E deficiency developed in the animals deprived of this vitamin after longer periods of feeding and the results of these experiments will be the subject of a later report.

After 335 days of feeding and again after 512 days, blood samples were drawn and placed in heparinized tubes. The erythrocytes were spun down and washed several times with isotonic saline, frozen, and packaged with dry ice prior to analysis. Lipid extracts were prepared as described by Horwitt et al. ('59). Samples were analyzed on ethyleneglycol succinate polyester columns using a tritium cell as the detector. Assignments of structure are based on the data of Farquhar et al. ('59) and Witting et al. ('61) and use of the carbon number method suggested by Woodford and van Ghent ('60). To inhibit peroxidation, 3 mg of tocopherol were added to each sample at the initiation of lipid extraction. Fatty aldehydes were determined as the free aldehydes on the same chromatographic traces as the fatty acid methyl esters. The resulting percentage of aldehyde was 95% of the quantity determined in the original intact lipid by treatment with 2,4- dinitro- phenylhydrazine.

RESULTS AND DISCUSSION

The first sign of fat deficiency in the monkeys fed the low-fat diet was a generalized dryness and scaliness of the skin, particularly noticeable on the face, abdomen and tail. The skin turgor was poor and the hair appeared more brittle than normal. After the diet was supplied for about 200 days, there was a gradual loss of hair from the face and ventral portion of the body. In two of the more severely affected monkeys, almost all of the hair was lost but after several months one of these monkeys regained most of its hair while still receiving the low-fat diet. One of the monkeys exhibiting the most severe fat-deficiency syndrome received vitamin E, whereas the other monkey received only the vitamin E-free diet and both of the animals weighed less than 2 kg at the beginning of the experiment. In figure 1 is shown a photograph of a fat-deficient monkey that had received the low-fat diet for approximately 550 days compared with a control monkey fed the diet containing lard and cod liver oil for the same period of time. The early signs of fat deficiency in the rhesus monkey are similar to the description of Portman et al. ('59) of the signs which developed when *Cebus* monkeys were fed a fat-free diet for a 35-week period.

Fat deficiency did not appreciably impair the growth of the monkeys in these experiments. The average daily weight gain between the 200th and 400th days was 4.1 and 3.9 gm per day while the monkeys were fed the low-fat diet and the lard and cod liver oil-containing diet, respectively. The explanation for this observation, which is contrary to observations in other species is not readily apparent. It is possibly related to the composition of the depot fat prior to beginning these experiments and to the rate of turnover of depot fat in the monkey, neither of which were measured. It is estimated that the corn starch in the fat-free diet supplied approximately 100 mg of linoleic acid per 100 gm of diet, and how much this small amount contributed to the maintenance of the rate of growth is not known.

The fatty acid composition of the lipids from erythrocytes of monkeys fed diets with and without fat are shown in table 1. There were considerable differences in the fatty acid composition of erythrocyte lipids as a result of the presence or exclusion of dietary fat. Some of these differences are related to the inclusion of cod liver oil in the diet. Note the high level of the more unsaturated fatty acids in the group receiving the combination of lard and cod liver oil. The presence of 10 to 12% of pentaenoic acids and 10% of docosahexaenoic acid is most likely due to direct incorporation of these fatty acids from the dietary cod liver oil and it is possible that the lower level of oleic, palmitoleic and arachidonic acids in these animals is related to the high content of the more unsaturated fatty acids. It has also been

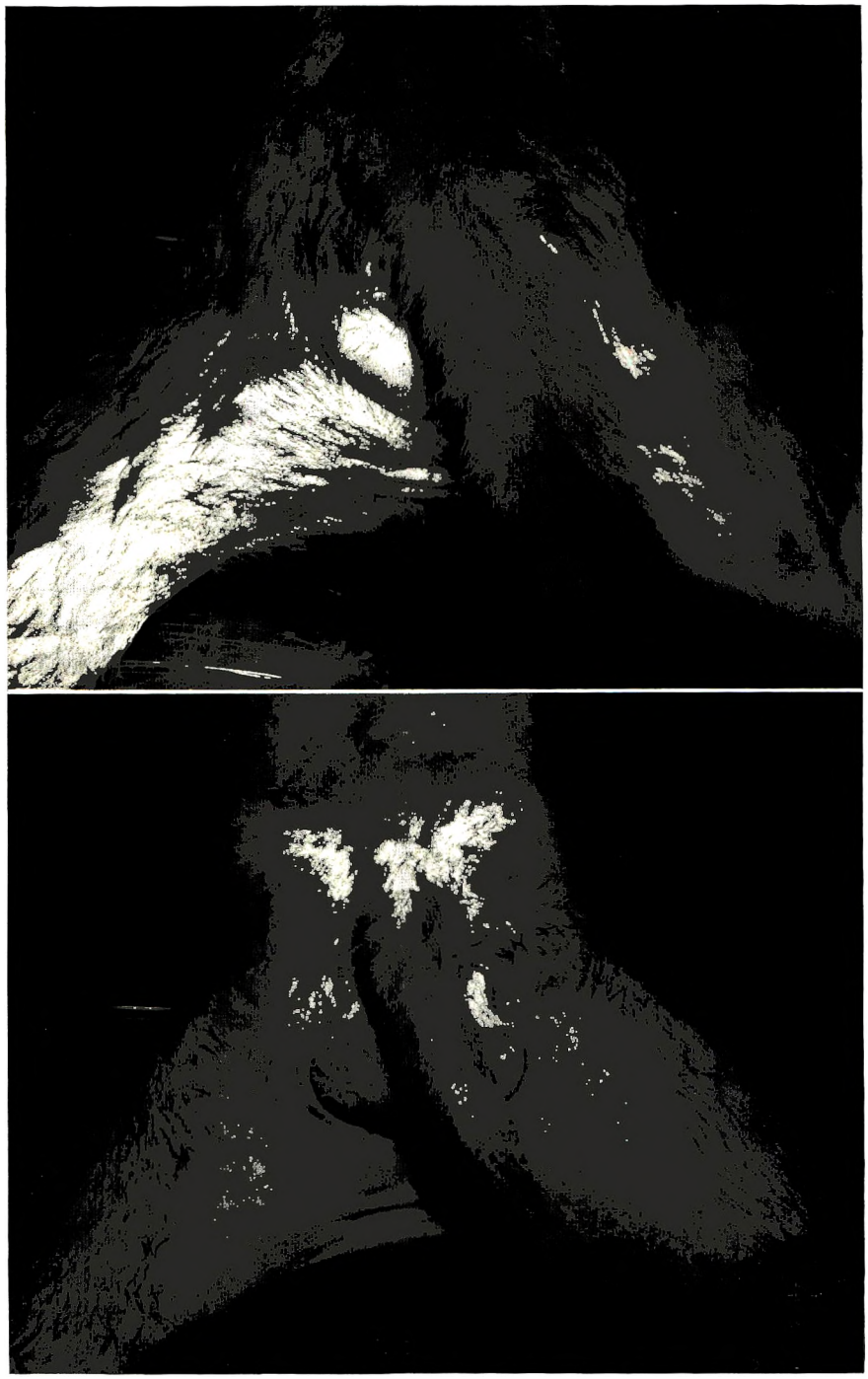

Fig. 1 Photograph of monkey fed a fat-containing diet (upper) and a low-fat diet (lower). Note the loss of hair from the monkey that was fed the low-fat diet.

TABLE 1

Fatty acid composition of erythrocytes of monkeys fed diets with and without fat[1]

Fatty acid	After 335 days of feeding				After 512 days of feeding			
	Without dietary fat		With dietary fat		Without dietary fat		With dietary fat	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Myristic	0.7[2]	0.6– 0.7	0.3	0.2– 0.4	0.5	0.1– 1.0	0.4	0.3– 0.5
Palmitic	15.2	12.3–17.2	15.6	12.8–19.1	22.1	20.2–25.5	20.2	19.1–22.8
Palmitoleic	2.5	1.8– 3.3	0.5	0.3– 0.5	2.8	2.1– 3.4	0.2	0.2– 0.3
Stearic	10.5	9.9–11.9	15.2	14.1–17.1	9.8	8.9–10.9	15.1	13.7–16.6
Oleic	20.5	17.1–23.9	15.7	14.2–16.9	26.2	25.4–28.1	14.4	10.0–16.0
Linoleic	7.3	6.3– 8.1	11.0	8.9–12.9	6.0	5.4– 6.6	10.3	9.4–11.4
Arachidonic + trace of behenic	18.7	17.9–20.0	12.3	11.6–13.4	16.1	15.5–16.3	12.5	11.0–13.8
C_{20} Triene 5, 8, 11	4.8	4.3– 5.5	0.2	Tr[3]– 0.4	5.3	3.0– 7.2	0.1	Tr– 0.3
C_{20} Triene	3.3	2.8– 4.0	0.8	0.6– 1.3	2.1	1.6– 2.7	0.8	0.6– 1.2
C_{20} Pentaene	1.8	0.8– 3.4	7.5	6.3– 9.4	1.6	1.3– 1.7	7.5	5.6–11.6
C_{22} Pentaene	1.5	1.1– 1.9	4.2	3.3– 4.8	0.6	0.3– 0.9	3.2	2.5– 4.6
C_{22} Hexaene	1.4	0.8– 2.1	10.8	8.5–12.4	0.5	0.4– 0.6	10.5	8.2–15.1

[1] Six monkeys received the diet containing fat and 4 monkeys received the low-fat diet; the diet with fat contained 8% of lard and 3% of cod liver oil.

[2] Data are expressed as per cent of total fatty acids.

[3] Tr indicates Present, but less than 0.05%.

observed that the arachidonic acid content of structural and vital lipids of rats is drastically depressed when cod liver oil is included in the diet (Witting et al., '61).[5]

As has been noted in various tissues of other animals (Aaes-Jørgensen, '61), the concentration of eicosatrienoic acids is much higher in the monkeys that were not fed unsaturated fatty acids. Fulco and Mead ('59) found a 11:1 ratio of Δ5, 8, 11 eicosatrienoic acid to Δ7, 10, 13 eicosatrienoic acid in the essential fatty acid-deficient rat. In the present experiments on monkeys, the ratio of the 5, 8, 11 isomer to the other trienoic acid was either 10:7 or 10:4 depending on the time period taken. The positions of the double bonds in the second isomer are not known but both isomers yield conjugated trienes by alkali isomerization.

An increase in the concentration of eicosatrienoic acid in erythrocyte lipids from monkeys deprived of essential fatty acids has previously been observed.[6]

There was no difference in the concentration of fatty aldehydes as a result of the variation in the fat content or vitamin E content of the diet. The principal aldehydes were hexadecanal and octadecanal which together accounted for about 2% of the total extractable lipid in each group.

SUMMARY

Young rhesus monkeys were fed purified diets containing 8% of lard and 3% of cod liver oil or the same diet with the fat omitted for 550 days. The animals fed the low-fat diet developed symptoms of fat deficiency which included scaliness and dryness of the skin, and loss of hair. The fatty acid composition of the erythrocytes was considerably different in the two groups of monkeys. The erythrocytes from the animals that received the low-fat diet contained higher concentrations of palmitoleic, oleic, and arachidonic acids and a lower concentration of linoleic acid.

[5] Century, B., L. A. Witting, C. C. Harvey and M. K. Horwitt 1961 Changes in fatty acid composition of skeletal muscle of rats induced by varying the lipid in the diet. Federation Proc., 20: 367 (abstract).

[6] Greenberg, L. D., and H. D. Moon 1960 Eicosatrienoate in the erythrocytes of monkeys deprived of essential fatty acids. Federation Proc., *19*: 321 (abstract).

The concentration of eicosatrienoic acids was high in the erythrocytes of these monkeys, whereas high concentrations of docosahexaenoic acid and C_{20-22} pentaenoic acids were noted in the erythrocytes from the monkeys given the diet that contained cod liver oil. These results emphasize that the fatty acid composition of structural lipids may be significantly influenced by the fatty acid content of the diet.

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Major Dietary Constituents and Vitamin B_{12} Requirement[1]

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Deficiency of vitamin B_{12} in the maternal diet results in weakness, emaciation and high mortality among the offspring (Schultze, '49; Lepkovsky et al., '51; Jaffé, '56). The incidence of congenital malformations, such as hydrocephalus, eye abnormalities and bone defects, is also increased (O'Dell et al., '51; Grainger et al., '54).

Up to the present time the effect of diet composition upon vitamin B_{12} requirement has been investigated chiefly by means of growth studies. Hartman et al. ('49) as well as Peterson and Register ('58), reported a more severe state of deficiency in growing rats fed diets high in protein. Diets high in fat spared the vitamin B_{12} requirement in growing mice (Bosshardt et al., '50) but Spivey Fox et al. ('56) observed that in the chick high levels of dietary fat increased the vitamin B_{12} requirement. McCollum and Chow ('50) reported that diets high in carbohydrate produced a more pronounced deficiency in female rats than in littermate males. Cuthbertson and Thornton ('52) have shown that rats have a higher vitamin B_{12} requirement when lactose rather than glucose served as the source of carbohydrate.

The present investigation was initiated to develop the most effective maternal diet for production of vitamin B_{12} deficiency in the offspring. The effects of high levels of protein and fat, as well as the source of carbohydrate on the vitamin B_{12} requirement, were studied. The criteria of adequacy were growth rate of weanling rats, the reproduction and lactation performance of mature depleted females, and the incidence of congenital abnormalities.

METHODS

Compositions of the rations used are shown in table 1.[2] Ration A, which contained 30% of isolated soy protein and 10% of soybean oil, served as the basal diet since it contained the more commonly used balance of protein, carbohydrate and fat. Ration B, the high-protein diet, contained 60% of the protein and ration C, the high-fat diet, contained 30% of soybean oil. Each diet was fed with and without added cobalt. Since the added cobalt supplement had no significant effect on the incidence of congenital malformations or on the reproduction and lactation performance, the data on diets with and without added cobalt were combined. Lactose was substituted for sucrose in ration D and glucose was the carbohydrate source in ration E. For the positive control diet cyanocobalamin was added to the basal diet (A) at a level of 0.003 mg per 100 gm.

Albino rats of the Wistar strain were used. For the growth studies, weanling (about 30 days of age) littermates, produced by dams fed a vitamin B_{12}-deficient diet, were used. They were paired as to sex and weight and were caged individually. One of each pair was fed the deficient ration and the other the corresponding vitamin B_{12}-supplemented ration. Ten females and 10 males, with an average initial weight of 43 and 42 gm, respectively, were supplied with each of the three diets, basal, high-protein, and high-fat, with and without added vitamin B_{12}. Food and water were consumed ad libitum during the 4-week experimental period.

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[2] The authors wish to acknowledge gifts of vitamins from Merck and Company, Rahway, N. J., American Cyanamid Company, Pearl River, New York and Hoffmann-La Roche, Nutley, New Jersey.

TABLE 1

Composition of rations

Constituents	Ration				
	A	B	C	D	E
	%	%	%	%	%
Isolated soy protein[1]	30.0	60.0	30.0	30.0	30.0
Sucrose	54.6	24.6	34.6	—	—
Lactose	—	—	—	53.6	—
Glucose hydrate	—	—	—	1.0	54.6
Salts mix[2]	5.0	5.0	5.0	5.0	5.0
Soybean oil	10.0	10.0	30.0	10.0	10.0
DL-Methionine	0.3	0.3	0.3	0.3	0.3
Choline Cl	0.1	0.1	0.1	0.1	0.1
Vitamin supplement[3]	+	+	+	+	+

[1] Alpha Protein obtained from Central Soya Company, Chicago, and purified as described by O'Dell et al. ('52). By analysis this product contained 90% crude protein, 1.0% cystine and 1.0% methionine.

[2] Salts mixture of Richardson and Hogan ('46) except that cobalt was omitted. Approximately one-half of the animals in the reproduction and lactation study received a cobalt supplement. By analysis ration A contained approximately 7 parts/billion of cobalt.

[3] The following vitamins were supplied per 100 gm of diet: (in milligrams) *dl*-α-tocopheryl acetate, 3.0; menadione, 1.0; thiamine·HCl, 1.6; riboflavin, 1.6; pyridoxine·HCl, 1.6; Ca pantothenate, 4.0; biotin, 0.02; folic acid, 0.5; and vitamin A, 2000 IU and vitamin D_3, 280 IU. When fed, vitamin B_{12} was added at the level of 0.003 mg.

TABLE 2

Effect of dietary protein and fat on the growth response to vitamin B_{12}

Ration	A (basal)		B (60% protein)		C (30% fat)	
Vitamin B_{12} supplement	—	+	—	+	—	+
Gain of males, gm	98 ± 10.0[1]	129 ± 6.8	63 ± 7.4	112 ± 6.7	76 ± 7.2	114 ± 6.4
Difference in gain, gm	31		49		38	
Gain of females, gm	78 ± 4.8	88 ± 4.5	63 ± 6.3	81 ± 2.4	87 ± 4.2	99 ± 3.3
Difference in gain, gm	10		18		12	

[1] Standard error of the mean.

TABLE 3

Analysis of variance of data presented in table 2[1]

Source of variation	Degree of freedom	F values, rations A and B[2]	F values, rations A and C[2]
Sex (S)	1	24.58[3]	13.44[3]
Protein (P)	1	16.05[3]	—
Fat (F)	1	—	0.91
Vitamin B_{12}	1	34.65[3]	26.21[3]
S × P	1	2.63	—
S × F	1	—	10.03[3]
S × vitamin B_{12}	1	8.11[3]	7.06[3]
P × vitamin B_{12}	1	2.04	—
F × vitamin B_{12}	1	—	0.24
P × vitamin B_{12} × S	1	0.33	—
F × vitamin B_{12} × S	1	—	0.09

[1] Snedecor ('56).

[2] Includes only data from rations A and B and A and C, respectively.

[3] Significant at the 1% level.

Females used in the reproduction and lactation study were depleted of vitamin B_{12} by maintaining them from weaning to maturity with ration A or with a similar diet based on soybean meal (O'Dell et al., '51). At maturity (about 160 to 180 gm) groups of 5 females and one stock colony male were housed in suspended cages and fed ad libitum. The experimental period varied from three to 12 months, but the average length of time fed the respective diets was 10 months. In late pregnancy the females were transferred to individual cages where the litters were born. They were returned to the breeding cage immediately after the litter was removed. At birth the offspring were examined for hydrocephalus, eye defects, cleft palate and other gross abnormalities and about one-half of the litters was cleared by the technique of Dawson ('26) for examina-

tion of the skeleton. The other litters were allowed to nurse in order to determine viability of the offspring and the lactation performance of the dams.

RESULTS

Results of the growth studies are summarized in table 2. An analysis of variance of the data (table 3) shows that each of the factors, sex, protein and vitamin B_{12}, had a significant effect on growth. There was also a significant interaction between sex and vitamin B_{12}. The growth response due to vitamin B_{12} was greater for the males than for the females with all diets. Separate analysis showed, however, that the response of females alone to vitamin B_{12} was statistically significant. Although the growth response of both sexes to vitamin B_{12} was considerably greater with the high-protein diet, than with the basal, statistically there was not a significant interaction between vitamin B_{12} and protein. The interaction between vitamin B_{12} and fat was even less. This failure to achieve statistical significance probably reflects largely the high variability in growth rate. High protein was detrimental to growth both in the presence and absence of vitamin B_{12}.

In table 4 are shown the reproduction and lactation performance of the females and the incidence of congenital malformations among their offspring. The weaning percentage with the vitamin B_{12}-supplemented diet was 61% and the average weight at weaning was 55.6 gm. The incidence of congenital abnormalities was low except that the percentage of bone malformations was higher than occurs

TABLE 4

Effect of dietary protein and fat on the vitamin B_{12} requirement as measured by reproduction, lactation and incidence of congenital malformations

	Rations			
	A (basal)	B (60% protein)	C (30% fat)	A + vitamin B_{12}
	Reproduction and lactation performance			
No. of females	144	53	44	40
No. born	2275	688	932	713
Stillbirths, %	14.1	7.7	10.5	5.2
Weaned at 4 weeks, %	7.6(964)[1]	8.2(159)	17.2(244)	61.2(297)
Weaning weight, gm	42.7(73)	38.3(13)	51.0(42)	55.6(182)
	Congenital abnormalities			
Hydrocephalus, %	8.7	5.1	7.1	0.1
Eye abnormalities, %	5.5	4.1	4.6	0.8
Bone malformations, %	16.8(1341)	14.1(581)	15.5(716)	10.0(90)

[1] Numbers in parentheses refer to number of observations on which the percentage is based.

TABLE 5

Effect of carbohydrate source on the vitamin B_{12} requirement as measured by reproduction, lactation and incidence of congenital malformations

	Rations		
	A (sucrose)	D (lactose)	E (glucose)
	Reproduction and lactation performance		
No. of females	144	20	27
No. born	2275	184	551
Stillbirths, %	14.1	21.7	9.6
Weaned at 4 weeks, %	7.6(964)[1]	0 (75)	0 (183)
Weaning weight, gm	42.7(73)	—	—
	Congenital abnormalities		
Hydrocephalus, %	8.7	1.6	8.2
Eye abnormalities, %	5.5	3.2	2.5
Bone malformations, %	16.8(1341)	22.8(35)	20.6(253)

[1] Numbers in parentheses refer to number of observations on which the percentage is based.

with our colony diet of natural feedstuffs. The bone defects most commonly observed with all diets were irregularities of the sternebrae.

Comparison of rations A and B indicates that high protein had only a slightly adverse effect on the lactation performance and it did not increase the incidence of congenital abnormalities. The reproductive rate with the high-protein diet was subnormal and many of the animals had diarrhea and appeared unkempt. Of the vitamin B_{12}-deficient diets, the best reproduction and lactation performance occurred with the high-fat diet, but the percentage of malformed offspring was not significantly affected.

The performance of dams fed diets containing sucrose (A), lactose (D) and glucose (E) is summarized in table 5 and shows that lactose depressed the rate of reproduction, and increased the percentage of stillbirths. None of the offspring survived, but if anything, lactose decreased the incidence of hydrocephalus. Females fed the glucose diet failed to wean their offspring, and in most respects gave results comparable to those with the sucrose diet.

DISCUSSION

Diets high in soy protein were deleterious to rats as measured by growth, reproduction or lactation. The growth response obtained from vitamin B_{12} supplementation of the high-protein diets was greater than with any other, but because of wide variation the interaction was not statistically significant. Likewise, the incidence of congenital malformations was no higher with the high-protein diet deficient in vitamin B_{12} than with the more usual protein level. These results do not agree with those of Hartman et al. ('49) and Peterson and Register ('58) who observed more severe deficiencies with high-protein diets.

The effect of dietary fat on vitamin B_{12} requirement is not clear inasmuch as Bosshardt et al. ('50) reported a sparing action in mice and McCollum and Chow a similar effect in rats, whereas Spivey Fox et al. ('56) observed that fat increased the requirement in chicks. In this study high fat had no significant effect on the vitamin B_{12} requirement as measured by the growth rate of weanling rats. When reproduction and lactation were the criteria, fat appeared to have a slight sparing action, but it did not affect the incidence of congenital anomalies. Thus, there seems to be a species difference in that fat increases the vitamin B_{12} requirement of chickens, but tends to decrease it in mammals. This may be related to the relative methionine requirements of two species and to the basal diets used. It should be pointed out, too, that substitution of fat for carbohydrate in the diet decreases the percentage of protein calories. This might explain part of the fat-sparing effect when protein is not adjusted.

When lactose replaced sucrose as the source of carbohydrate in this study, it had an adverse effect on reproduction but did not increase the incidence of malformed offspring and consequently was less useful in a diet for study of the effect of vitamin B_{12} deficiency on congenital abnormalities. Since lactose was not fed in a diet supplemented with vitamin B_{12}, it is not clear whether its deleterious effect on reproduction was caused by its effect on vitamin B_{12} requirement or by some other mechanism.

From the observations made, it may be concluded that the basal diet was best adapted for production of vitamin B_{12} deficiency in the reproducing female rat. There was no advantage in using extremely high levels of protein or fat, and lactose was no better for this purpose than sucrose or glucose. Even though the basal diet was low in cobalt (10 parts per billion), the addition of cobalt had no demonstrable effect on the reproductive response to vitamin B_{12}. Even this level, however, supplies far more cobalt than would be required for biosynthesis of the vitamin requirement. Nevertheless, it seems advisable to keep the dietary cobalt level as low as possible for production of vitamin B_{12} deficiency.

SUMMARY

The effects of the concentration of two major dietary constituents, protein and fat, and of the type of carbohydrate on the vitamin B_{12} requirement of the rat were investigated. The criteria of adequacy of the diets were growth rate of weanling rats, reproduction and lactation performance of mature females and the incidence of gross malformations among the offspring. The

basal diet contained sucrose, 10% of fat and 30% of soy protein. One diet contained 60% of soy protein, one 30% of fat, one lactose and one glucose. All supplements were added at the expense of sucrose.

The high-protein and high-fat diets slightly increased the growth response to vitamin B_{12}, but the interaction was not statistically significant. High protein decreased reproduction but did not increase the incidence of congenital anomalies. High fat tended to improve the reproduction and lactation performance in the absence of vitamin B_{12} but had no effect on congenital malformations.

Glucose gave results entirely comparable to those obtained with sucrose. In the absence of vitamin B_{12}, lactose impaired reproduction but tended to decrease the incidence of abnormal offspring.

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Feeding of Rats with Phenylacetic Acid: Organic Acids in Urine and Tissues

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In view of the often quoted (Woolf, '51; Meister, '57) but unconfirmed observation of Sherwin and Kennard ('19) that phenylacetic acid exerts a toxic effect on the nervous system, and because phenylacetic acid is well known to be one of the abnormal metabolites excreted in the urine by phenylketonuric patients (Knox and Hsia, '57), it was considered worthwhile to explore both the clinical and some relevant biochemical effects of prolonged administration of this compound on young rats. In the rabbit, horse, sheep, monkey, and dog, phenylacetic acid has been known to be excreted as phenylacetylglycine (Williams, '47). It was therefore intended to determine (1) whether any neurological signs developed in the animals, and (2) whether the phenylacetic acid was accumulated in the tissues or was excreted rapidly, and if it was detoxicated in the rat also, largely through conjugation with glycine to form phenaceturic acid. In view of the possibility of disturbance in cerebral oxidative metabolism under the stress of circulating phenylacetate, it was decided to apply a method of chromatography that would reveal both the conjugation compounds and the Krebs cycle acids.

EXPERIMENTAL

Two groups of 4 rats each, 6 months and 4 weeks old, respectively, were given intraperitoneal injections of phenylacetic acid in doses of 2 and 5 mmoles per kg of body weight (272 and 680 mg per kg) respectively, three times a week for one to 7 months. Urine samples were collected regularly (by keeping the rats in metabolic cages) from these and from the two control animals in each group, that were given intraperitoneal injections of distilled water. At the end of the period of observation, most of the animals were sacrificed and their tissues collected for analysis (table 1).

METHODS

For urine. The creatinine level of each urine specimen was first estimated, applying Jaffe's reaction and Folin's method (Hawk et al., '49).

The procedure then used was a paper chromatographic identification of organic acids, including phenylic compounds, Krebs cycle intermediates and some other acids, as described originally by Nordmann et al. ('54a,b). In brief, the following steps were carried out: urine equivalent to 1.0 mg of creatinine was passed through columns of anion exchange resin (prepared as advised by the authors), followed by distilled water to wash off the salts and amino acids, and then by a 12 M solution of formic acid to obtain the eluate of organic acids. The latter was divided into halves, dried at room temperature, redissolved in 50% alcohol, and spotted on Whatman no. 1 paper. Duplicate chromatograms were thus prepared and developed through ethanol-ammonia-water and, after drying, through propanol-eucalyptol-formate, in two directions. Staining with 0.15% solution of bromcresol green revealed yellow acid spots in a greenish-blue background. The chromatograms were immediately preserved in cellophane "Kodapak sleeves,"

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TABLE 1

Clinical protocol of experimental and control rats

	Rat no.	No. of days	No. of injections	Dose/ injection	Total dose	Weight at start	Weight at termination	Gain in weight
					gm	*gm*	*gm*	*gm*
Experimental	1	63	43	2 mmole/kg of phenylacetate	2.654	163	278	115
	2	63	43	2 mmole/kg of phenylacetate	2.366	162	254	92
	3	137	94	2 mmole/kg of phenylacetate	7.208	195	342	147
	4	32	23	2 mmole/kg of phenylacetate	1.365	176	243	67
Control	5	58	40	Distilled water	—	184	378	194
	6	135	92	Distilled water	—	169	341	172
Experimental	7	31	24	5 mmole/kg of phenylacetate	2.304	96	200	104
	8	31	24	5 mmole/kg of phenylacetate	1.782	82	138	56
	9	192	125	5 mmole/kg of phenylacetate	15.871	79	215	136
	10	192	126	5 mmole/kg of phenylacetate	15.137	85	225	140
Control	11	31	24	Distilled water	—	83	206	123
	12	192	127	Distilled water	—	79	233	154

and the density of spots was expressed semi-quantitatively by gross visualization, along a scale of 0.5 to 4 in the increasing order of intensity.

For tissues. Animals were killed by drawing their heart blood, and then portions of brain, liver, kidney, spleen and muscle were removed quickly and transferred to previously weighed vials containing 80% ethanol. After obtaining the wet tissue weights, more alcohol was added in the proportion of 1 gm of tissue:10 ml EtOH, and the tissues were then homogenized thoroughly in a small stainless steel homogenizer. These alcoholic extracts were then centrifuged and the filtrates kept in the cold. The alcoholic filtrate was passed through the Dowex-2 column for desalting. Both this and the subsequent organic acid chromatography, were carried out in a manner identical to that used for urine, except that for adequate visualization of the constituent organic acids, eluate equivalent to 0.7 gm of a tissue or 7.0 ml of blood was deposited on each chromatogram.

The identification of the compounds discussed below was achieved not only by the position of their spots on the chromatograms, but (1) by running standard solutions of the suspected compounds together with the urine or tissue extract, and (2) by elution of the spot of the suspected compound off the fully developed chromatogram and rechromatographing the eluate in one of the two solvents against various authentic solutions. In a few cases quantitative evaluation of the amount of acid in a spot was carried out by a potentiometric titration of its eluate against a very weak solution of sodium hydroxide, using a micro-pipette-burette. Using the same method, recovery experiments were also conducted on eluates of a number of spots of phenylic and Krebs cycle compounds containing known amounts of authentic solutions, to assess the loss in chromatography of these compounds. In general,

Krebs cycle acids were recovered much more than the phenylic acids and conjugates.

RESULTS AND DISCUSSION

Frequent clinical examination of both the groups of experimental animals—those given 2 mmoles per kg of phenylacetic acid, from the age of 6 months (group 1), and those given 5 mmoles per kg from the age of 4 weeks (group 2)—failed to reveal at any stage any neurological deficit. Their gait, co-ordination and appearance, as also their feeding habits and gain in weight were alike comparable to those of the control animals of both these groups (table 1). Their activity in the cage and outside was quite normal. With 4 animals caged together, their social behavior was also observed to be unaffected after several months.

Chromatography for *organic acids in urine* revealed some interesting features. First, in the urine of experimental animals, there was invariably a smaller or larger amount of phenylacetic acid. It was detected as a spot above that of hippuric acid, in the system of chromatography used, and was identified by comparison of uni- and bidirectional movement of an eluate of this spot with that of a standard solution of phenylacetic acid. In the urine of control animals there was only rarely any trace of any spot above that of hippuric acid (compare figs. 1 and 2; and table 2). There was a possibility of the presence of some *p*-hydroxyphenylacetic acid being also present in the spot of phenylacetic acid, since these two acids are not adequately separable by the system of chromatography used (Nordmann et al., '58).

TABLE 2

Organic acids in urine of experimental rats: comparison with control rats

Rat no.	Date	Days on injection	Phenylacetic	Hippuric and phenylacetylglycine	Lactic	Pyrrolidone carboxylic	Glucuronic	Citric	Malic	α-Ketoglutaric	Succinic	Aconitic
1–4	18–4–57	1	1[1]	4	—	3	0	1	0.5	0.5	2	4
1–4	20–4–57	3	2	4	1	1	—	2	1	2	1	4
1–4	29–4–57	12	2	3	2	2	2	2	1	1.5	2	2
1–4	17–5–57	30	3	5	1	1	0.5	2	1	1.5	2	4
1–4	23–5–57	36	2	5	1	3	0.5	—	—	—	—	4
1–4	30–5–57	43	2	1.5	1	2	—	1	1	1	2	3
1–4	6–6–57	50	3	3	3	3	1.5	2	1.5	1	2.5	4
1–3	19–6–57	63	2	3	1	2	2	2	1	1.5	2	3
3	25–6–57	69	2	3	1	2.5	0.5	1.5	1	1	1.5	3.5
3	23–7–57	77	1	3	0.5	2	—	2	0.5	0	2	3
3	25–7–57	99	2	2	1	2	1	2	1	1	2	3
7–10	13–6–57	3	3	3	0.5	2	0.5	1	—	0.5	1	3
7–10	27–6–57	17	3	3	1	2	2	1	1	1	2.5	3
7–10	3–7–57	17	2	3	1.5	2	1	1	1.5	1	2.5	2.5
7, 8	11–7–57	31	2	3.5	1.5	3	2	1	0	0.5	1.5	1.5
9, 10	11–7–57	31	2	2	2	3	1.5	2	1	2	1	3
7, 8	17–7–57	37	2	3	1.5	2	1	1	0	0	1.5	1.5
9, 10	17–7–57	37	1	1	0.5	1	2	2	1	1	1	2
9, 10	2–8–57	52	2	2	2	3	2	0	2	2	2	3
9, 10	13–9–57	92	2	4	0	2	0.5	2.5	1.5	1	1	3
9, 10	27–9–57	106	2	3	2.5	2.5	—	2.5	1	0.5	1	3
Average "value" for 21 observations on 8 experimental rats			2.2	3.0	1.2	2.1	1.20	1.5	0.8	1.0	1.6	3.0
Average "value" for 15 observations on 4 control rats			0.13	1.3	0.9	1.4	1.07	1.8	0.8	0.8	1.5	2.6

[1] Figures in this table and the next express the approximate comparative "values" of any given organic acid assessed on gross visualization of its spot in a pair or a series of duplicate chromatograms.

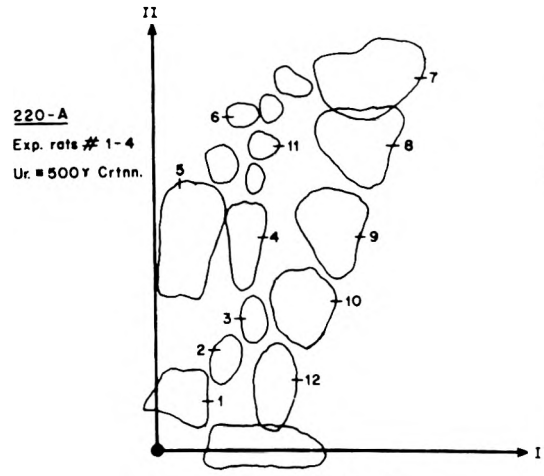

Figure 1

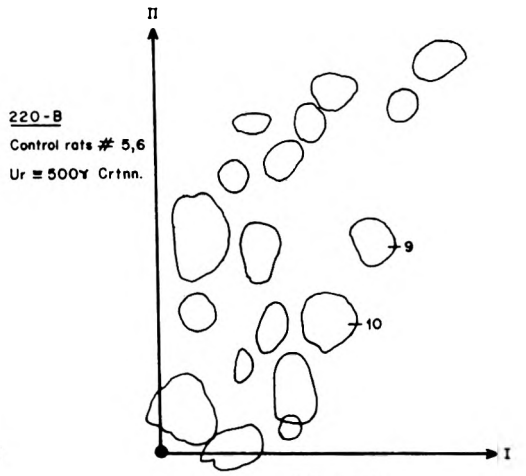

Figure 2

Figs. 1 and 2 Urinary organic acids. (1) Rats fed phenylacetic acid. 1–Citric, 2–malic, 3–α-keto-glutaric, 4–succinic, 5–acotinic, 6–fumaric acid; 7–phenylacetic acid, 8–hippuric and phenaceturic acids; 9–lactic, 10–pyrrolidone carboxylic, 11–glutaric acid, 12-glucuronide. (2) Control rats; note almost total absence of spots 7 and 8, and persistence of the dense spot of aconitate.

The second interesting feature was a marked increase in the combined excretion of phenylacetylglycine (phenaceturic acid) and hippuric acid in the urine of experimental rats (spot 8, fig. 1) as compared with the control animals. The paper chromatographic method used did not permit separation of these two acids. Their presence was identified by running standard solutions of hippuric acid and of phenylacetylglycine, both concurrently with the urine and separately with eluates of the spot. Hippuric acid was further identified by the Gaffney reaction, when on spraying the chromatogram (previously developed with bromcresol green) with *p*-dimethylaminobenzaldehyde and heating, it gave a bright orange color (Gaffney et al., '54). Phenylacetylglycine did not give this reaction.

It has long been recognized (Salkowski, 1885), that the liver of animals below the status of man or, at best, of primates, conjugates circulating phenylacetic acid with glycine, to produce phenaceturic acid. Hence a detoxication of injected phenylacetic acid and its subsequent excretion as phenylacetylglycine were physiologically expected events.

With respect to the excretion of hippuric acid, the increased presence of this compound in the urine of experimental rats may be more apparent than real. It was frequently detected in the urine of our control animals as well. In lower animals the metabolism of phenylacetic acid is qualitatively similar to that of benzoic acid. The synthesis of phenaceturic acid and of hippuric acid is carried out by two enzymes, the difference between which is related mainly to the nature of the side chains of the substrates. Benzoic acid and hippuric acid therefore represent merely two different stereochemical patterns for similar enzyme action (Williams, '47). In the rat, as in man, benzoic acid is a normally occurring metabolite, and some of its urinary benzoate (Schreier et al., '54) and hippurate (Armstrong et al., '55) are derived endogenously from phenylalanine. In the rat, then, both the conjugations with glycine take place readily. Although in our control animals the small common spot of these two conjugation products appeared to consist largely of hippuric acid, in the experimental rats the same common spot became much larger (figs. 1 and 2; table 2) owing almost entirely to the presence of phenylacetylglycine.

The urinary excretion of phenylacetate, as well as phenylacetylglycine, was noticed from the first day onwards after its injection, indicating very rapid detoxication and elimination. On visual comparison of the chromatograms, as well as on the basis of the few quantitative estima-

tions, it appeared that by far the larger amount excreted was in the form of the conjugated compound.

After a single dose of phenylacetic acid to man, about 95% is excreted as phenylacetylglutamine (the analogue of phenylacetylglycine in the rat) and the remainder in conjugation with glucuronic acid. On continued ingestion of the acid, there appears to be a tendency for more of the glucuronide to be excreted (Ambrose et al., '33). In the rabbit also, after a single dose of 1.5 gm, about 53% is excreted as phenaceturic acid, and 5% as the glucuronide (Bray et al., '46). A careful evaluation of the size of the glucuronide spot in our chromatograms of the control and the experimental samples of urine failed to reveal any consistent difference (table 2), suggesting that the predominant pathway for detoxication of phenylacetate was through conjugation with glycine. In individual cases, however, the glucuronide spot was extremely small in the control samples. In these instances the spot for hippuric acid was quite conspicuous, and vice versa, there being a sort of inverse relationship between these two compounds (see fig. 2).

Another noticeable feature of chromatography of rat urine for organic acids, was the very high level of excretion of aconitic acid, when compared with corresponding amounts of human urine or monkey urine (spot 5, figs. 1 and 2). There was *no* appreciable difference, with respect to this feature, between the experimental and control rats of the present experiment. On rough evaluation, the amount of aconitic acid excreted appeared to be about two to 4 times the amount of any of the other Krebs cycle acids detected in the urine by this method, such as citric, α-ketoglutaric, succinic, or malic acid.

Finally, a moderate degree of difference between experimental and control animals was seen to exist in their excretion of pyrrolidone carboxylic acid. This interesting compound about which so little is known, except that it is probably derived *in vivo* from D-glutamic acid, or, *in vitro*, upon boiling L-glutamic acid (Meister, '57), appears to be a normal constituent of blood and urine at least of man (Nordmann et al., '54, '58), of monkey[3] and of the rat. Glutamine, too, because of the great lability of its amide group, shows a tendency to cyclization to ammonium pyrrolidone carboxylate (Meister, '57). It is not inconceivable that this too contributes to the spot of pyrrolidone carboxylic acid in the system of chromatography used by us. Our experimental rats showed, as a group, a moderate and unaccountable increase in the excretion of pyrrolidone carboxylic acid than the control animals (spot 10, figs. 1 and 2).

A superficial but striking resemblance was noted between the organic acid chromatograms of rats administered phenylacetate and those of phenylketonuric patients, with two exceptions. Instead of phenylacetylglutamine, which was never observed in rats, phenylacetylglycine was observed and the spot for aconitic acid was much larger.[4]

Chromatography of *tissue extracts for organic acids* brought out a few worthwhile observations (table 3). In the first place, in none of the tissues examined, including the liver and blood, was there any detectable phenylacetic acid or phenylacetylglycine, despite the fact that the last injection of phenylacetic acid for any given animal was administered either the day previous to the day of sacrifice or on the same morning. This "absence" of phenylacetic acid in any of the tissues has to be understood with a slight modification in that the system of chromatography used is not very sensitive for phenylic compounds, which can be recovered only to an extent of 25 to 50% of the amounts spotted. It was calculated, however, that there was less than about 7 mg per 100 gm in tissues and less than around 1 mg per 100 ml in blood of either phenylacetic acid or of phenylacetylglycine.

Brain was the only tissue that showed the presence of N-acetylaspartic acid. This was detected as a large spot between spots of malic and pyrrolidone carboxylic acids (fig. 4). It was identified as such in the same system of chromatography by Du Ruisseau,[5] and has now been confirmed

[3] Dastur, D. K., unpublished observation.

[4] Dastur, D. K., and J.-P. Du Ruisseau, unpublished data.

[5] Du Ruisseau, J.-P., unpublished data.

TABLE 3

Organic acids in tissues of experimental and control rats

Rat no.	Number of observations	Tissue	Lactic	Pyrrolidone COOH	N-Acetyl-aspartic	Glyoxylic	Succinic	Malic	Fumaric	Aconitic	Citric
1,2,7,8	4	Blood	1.5	2.25	—	—	0.6	0.5	—	—	—
1,2,7,8	4	Brain	2.5	2.9	2.75	1.0	1.1	0.5	0.25	—	—
1,2,7,8	4	Liver	1.5	2.4	—	—	1.5	1.1	0.4	—	—
1,2,7,8	5	Muscle	1.7	2.2	—	—	1.3	0.6	0.6	—	0.2
1,2	2	Spleen	2.75	3.0	—	—	1.25	0.5	0.25	—	—
1,2	2	Kidney	1.75	2.5	—	—	1.5	1.25	0.25	1.0	—
2,8	2	Testis	1.5	3.0	—	—	0.75	0.75	—	—	0.5
					Controls						
5,11	2	Blood	2.5	3.0	—	—	0.5	0.5	—	—	—
5,11	2	Brain	2.75	3.25	3.25	1.5	1.0	0.25	1.0	—	—
5,11	2	Liver	2.5	3.0	—	—	1.75	1.5	0.5	—	0.5
5	1	Muscle	1.0	3.0	—	—	1.0	1.0	—	—	1.0
5	1	Spleen	2.5	3.0	—	—	1.0	1.5	1.0	—	—
5,11	2	Kidney	1.0	2.5	—	—	1.75	1.5	0.5	1.75	—

by the running of standard solutions. There was no appreciable difference in the amount of this compound in the brain of our experimental and control rats. The place of N-acetylaspartic acid in the metabolism of the brain remains unknown, although it has been observed in high concentrations (of 80 to 110 mg per 100 gm) in different species of mammals and birds (Tallan, '57).

Of the various tissues examined, *kidney* alone revealed the presence of aconitic acid (fig. 3). Very large amounts of this acid were detected in the urine: roughly about 20 times more in a chromatogram of urine in an amount the equivalent of 0.5 mg of creatinine than in a chromatogram of kidney the equivalent of 0.7 gm of wet tissue. There was no difference between the experimental and control animals in this respect. Since some of the succeeding members of the Krebs cycle—such as succinic, fumaric and malic acids—were also detected in renal tissue, one could either postulate a specific enzyme peculiarity at the level of aconitase, so that aconitic acid accumulates and is excreted in the urine; or a renal tubular phenomenon, wherein the usual reabsorption of aconitate present in the glomerular

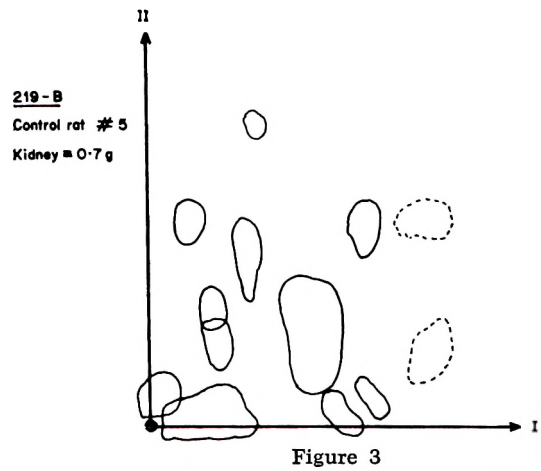

Figure 3

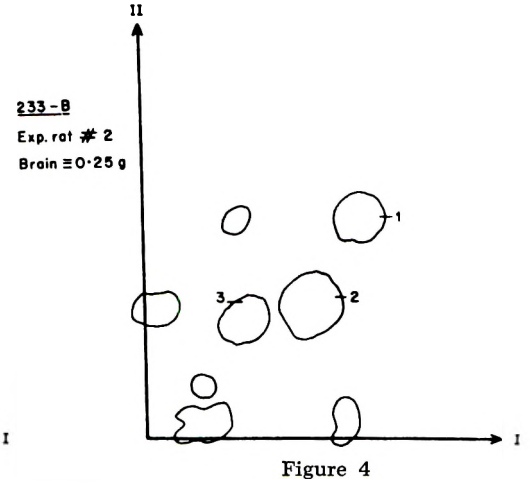

Figure 4

Figs. 3 and 4 (3) Organic acids in rat kidney homogenate; note clear presence of citric, malic, succinic, aconitic and pyrrolidone carboxylic acids. (4) Rat brain homogenate; in a much smaller amount of tissue, note clear presence of 1–lactic, 2–pyrrolidone carboxylic and 3–N-acetyl aspartic acids.

filtrate does not take place and large amounts of this acid are allowed to enter the urine. There was nothing remarkable in the presence or absence of any of the Krebs cycle intermediates in the other tissues examined (table 3).

In conclusion, observations on both young and old animals administered large quantities of phenylacetic acid over a prolonged period failed to reveal any abnormality of behavior, of neurological performance, or even of gain in weight as compared with the control animals (table 1). This fact, coupled with the absence of appreciable amounts of phenylacetic acid or of phenylacetylglycine in any of the tissues of the experimental rats, despite a continuous input of phenylacetic acid, suggests that in doses up to 5 mmole per kg, this acid fails to penetrate the blood-brain barrier to a significant extent, is detoxicated and excreted rapidly (mostly as phenaceturic acid), and is not in any way toxic to the otherwise normal recently weaned rat. The observations neither support nor contradict the earlier report of Sherwin and Kennard, which describes mostly the clinical picture of an acute intoxication with phenylacetic acid fed as a single dose of over 1.0 gm to dog, monkey and man. They rather lead one to doubt seriously the possibility of a small amount of circulating phenylacetic acid in phenylketonuric patients (Jervis, '54) being responsible for their mental and neurological changes, especially in view of the excretion of large quantities of phenylacetylglutamine by these patients (Woolf, '51). Bickel et al. ('55) did not observe any untoward effect of phenylacetic acid administration to 4 phenylketonurics, whereas the ingestion of additional phenylalanine or of phenylpyruvic acid was said to bring about a deterioration in their condition.

SUMMARY

1. Intraperitoneal injections of phenylacetic acid in doses up to 5 mmoles per kg of body weight were administered to two groups of rats consisting of adult and freshly weaned animals, for periods ranging from one to 7 months. Clinical condition and gain in weight were comparable to that of control animals injected with distilled water.

2. From the first day on, the urine of the experimental animals contained phenylacetic acid and a greater amount of combined phenaceturic and hippuric acid than the urine of control rats. The formation of these two conjugation compounds in the rat is discussed.

3. Blood, brain, liver, kidney, spleen, testis, or muscle of the experimental rats failed to reveal any appreciable amount of either phenylacetic acid or of phenylacetylglycine, indicating rapid detoxication and excretion of this acid, which appears to be nontoxic in the doses administered.

4. Rat urine was observed to contain exceptionally large amounts of aconitic acid. Neither the content of this nor of the other Krebs cycle acids was found to be affected by administration of phenylacetic acid.

ACKNOWLEDGMENT

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Effects of Antibiotics, Amino Acids, Zinc and Enzymes on the Growth Depression in the Turkey Produced by Raw Soybeans[1,2]

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Neither the mechanism of action of dietary antibiotics nor the growth depression resulting when unheated soybeans are substituted for properly processed soybeans are adequately explained. An effect common to both problems was reported by Borchers et al. ('57); that is, the inclusion of 0.1% of procaine penicillin and 0.1% of streptomycin sulfate into diets of rats, containing raw soybean meal, reversed the growth depression attributable to the meal. Since it has also been reported that amino acid supplementation can effectively reverse the raw soybean defect (Fisher and Johnson, '58; Borchers, '58), it is desirable to study the interrelationships between nutrients and antibiotics for the development of a more complete understanding of the raw soybean defect as well as the mode of action of antibiotics. The turkey appeared to be a good research animal for such a study, in view of its high protein requirement: 28% of the diet (National Research Council, '60).

The experiments to be reported here were designed to study the degree of the effect of ground raw soybeans (full-fat) on turkey poult growth, by substituting ground raw soybeans for equivalent amounts of solvent processed soybean meal and crude soybean oil. In addition, the effectiveness of antibiotics, zinc, methionine, and dietary enzyme sources upon the resulting growth depression was studied.

EXPERIMENTAL

Turkeys (Broad Breasted Bronze or White) were housed in electrically heated batteries with raised wire-mesh floors. Details of breed, sex, experimental period, and replication are given in table footnotes. Where a pretest period was used, poults were fed a complete turkey-starting diet, the formula of which is given in the thesis of Linerode.[4] The turkeys were carefully selected for each experiment, and random allotment procedures were used.

The composition of diet is given in table 1. Within each experiment, the soybeans, soybean meal, and crude soybean oil were obtained from the same production run.[5] Various analyses of the soybean products indicated that in ground soybeans the percentages of protein and fat were 37.1 to 37.6% and 17.2 to 18.0%, respectively. The protein level in the solvent processed soybean meal was 45.0 to 45.6%.

Procaine penicillin G was utilized as a product at 50% of potency, in which limestone served as the carrier. The other antibiotics were used in their crystalline forms.

The two heated ground soybean products were prepared as follows. The labora-

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[4] Linerode, P. A. 1960 Nutritional aspects of dietary antibiotics in turkeys with emphasis on raw soybean utilization. M S. Thesis. University of Minnesota Library.

[5] From Archer-Daniels-Midland Company, Minneapolis. The help of Gene Rabajdek in securing the samples is gratefully acknowledged.

TABLE 1
Composition of diets[1]

	A	B	C	D
	%	%	%	%
Soybean meal, solvent	56	39.6	27.3	0
Soybean oil, crude	12	8.4	5.7	0
Ground raw soybeans	0	20	35	68
Constant ingredients[2]	32	32	32	32

[1] By calculation, diet A contains 29.1% of protein and 876 Cal. of productive energy (Fraps, '46) per pound. Diets B, C, and D are isonitrogenous with A, and contain the same amount of oil from soybeans.

[2] The constant ingredients (in per cent): ground yellow corn, 16.1; fish solubles dried on soybean meal (100% equivalence), 3; alfalfa meal (17% protein, dehydrated), 3; dried whole whey, 3; dicalcium phosphate, 3; calcium carbonate, 2.75; iodized salt, 0.5; vitamin A (5000 IU/gm), 0.15; vitamin D_3 (3000 ICU/gm), 0.15; vitamin E acetate (44 IU/gm), 0.025; menadione sodium bisulfite—63% USP (4 gm/lb), 0.0125; vitamin supplement (4 gm riboflavin, 4 gm calcium pantothenate, 24 gm niacin, and 104 gm choline chloride/pound), 0.05; choline chloride (25%), 0.10; and vitamin B_{12} (20 mg/pound), 0.05.

In experiments 1, 3, and 4, trace mineral elements were added using Delamix (supplying diet with 60 ppm manganese, 20 ppm zinc, 20 ppm iron, 2 ppm copper, 1.2 ppm iodine and 0.2 ppm cobalt), Limestone Products Corporation of America, Newton, N. J., at 0.1% of the diet. In experiment 9, a trace mineral mixture was reconstructed using A.R. reagents $MnSO_4 \cdot H_2O$, $ZnCO_3$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, KI, and $CoCl_2 \cdot 6H_2O$. $CaCO_3$ served as the carrier and the mixture was added at the 0.1% dietary level. The same levels were employed excepting zinc, which was added at zero or 40 ppm of diet.

tory product was prepared by autoclaving the raw meal, spread in trays at a depth not greater than 5 cm, at 15 pounds pressure, for 20 minutes. With the commercial preparation, three tons of soybeans were crimped, placed in a rendering cooker at high heat, steam was injected until 50 pounds pressure was achieved, the pressure was released in 50 to 60 minutes, and the beans were cooled and ground.

Statistical analyses of variance were made for most experiments and reported by means of the Duncan's multiple range test (Snedecor, '56). Weights followed by the same letter are not significantly different at the 0.05 level of probability.

RESULTS

The effect of antibiotics in reversing the growth depression produced by diets containing high amounts of raw soybeans is shown in table 2. The antibiotics, notably streptomycin, streptomycin and penicillin, and erythromycin, produced larger percentage growth responses with the raw soybean than with the processed meal. Overall growth, however, was quite inferior to that of the controls, and it was apparent that the role of the antibiotics was only a minor one in counteracting the raw soybean toxicity.

A striking observation in addition to the slow growth rate when using 68% of raw soybeans was the development of a severe beak impaction problem (fig. 1). This occurrence may be related to the high water-solubility and cohesiveness on drying of raw soybean protein.

A series of trials was conducted with the objective of eliminating the beak impaction problem.[6] Effects of the following procedures were studied: (1) pressure pelleting of the diet; (2) screening fines from the soybean preparation; (3) soaking the feed with water; (4) adding 1% of mineral oil to the diet; and (5) starting experiments with older poults. The most successful procedure was to use poults that were one week of age when placed on experiment.

Prior to further studies on the dietary relationships involved in the growth depression of ground soybeans, it was essential to study the influence of dietary raw soybean level on growth. This relationship is indicated in figure 2. The growth depression was approximately linear when dietary percentage protein and body weight were plotted semi-logarithmically.

Due to the magnitude of the growth depression using diets containing high amounts of raw soybeans, subsequent studies on antibiotic and nutrient interrelationships were established including 20 or 35% levels of ground soybeans. The results of experiment 4, wherein the effects of penicillin and streptomycin were studied with zero, 20, and 68% of ground raw soybeans, is presented in table 3. In this study, the antibiotics essentially reversed the growth depression produced by 20% of raw soybeans, but demonstrated

[6] See footnote 4.

TABLE 2

Effect of type of soybean product and antibiotics on turkey poult growth and development of beak impactions (exp. 1)[1]

Kind of soybeans and supplements[2]	Observations, 14 days of age				
	Weight gain	Response	Efficiency	Mortality	Impactions[3]
	gm	%		%	%
Diet A (heated meal)					
Basal	150	—	0.58	7	0
0.1% Pen. and 0.1% strep.	166	10	0.64	0	0
0.1% Zinc bacitracin	168	12	0.58	0	0
0.1% Erythromycin thiocyanate	154	2.7	0.61	0	0
Diet D (68% raw meal)					
Basal	30	—	0.16	7	52
0.1% Pen.	32	6.7	0.25	0	52
0.1% Strep.	44	47	0.22	7	38
0.1% Pen. and 0.1% strep.	40	33	0.20	7	36
0.1% Sulfaquinoxaline	26	−13	0.10	14	44
0.1% Zinc bacitracin	36	20	0.22	7	49
0.1% Erythromycin thiocyanate	40	33	0.21	14	31
Diet D (68% raw flakes)					
Basal	27	—	0.26	21	35
0.1% Pen. and 0.1% strep.	34	26	0.39	36	28

[1] Each treatment consists of duplicate groups of 7 unsexed Broad Breasted White turkeys each. Data shown are averages of duplicates. Poults were placed on experiment at day of age; average weight was approximately 57 grams.

[2] Abbreviations: pen. is procaine penicillin G; strep. is streptomycin sulfate.

[3] Percentage impacted beaks indicates severity of beak impaction problem. Beaks were cleaned daily and figure shown represents the average of several days between 7 and 14 days.

Fig. 1 Illustration of beak impaction. The poult at the left is 7 days of age and has been fed a diet containing 68% of ground raw soybeans. Control at right.

only a partial (insignificant) effect with the high level of raw soybeans.

A series of experiments was conducted comparing the nutrient and antibiotic interrelationships involved in the raw soybean defect and these are reported elsewhere.[7] The essential observations of these experiments were repeated in experiment 9, which is reported in three tables for clarity of presentation.

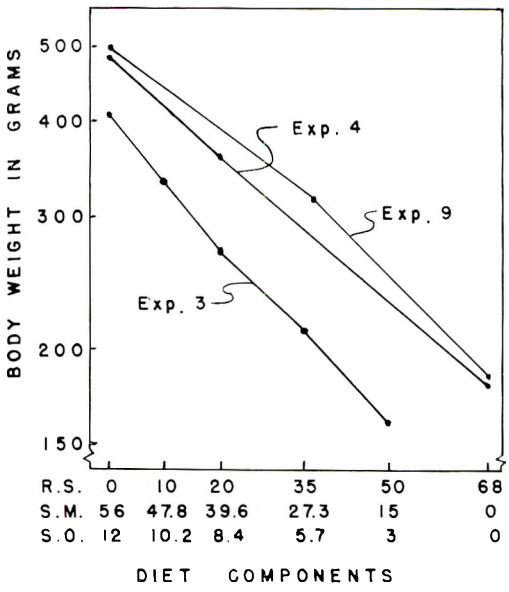

Fig. 2 Semi-logarithmic plot showing effect of raw soybean level on 4-week weights of turkeys. R.S. indicates raw soybeans; S.M., indicates soybean meal; S.O., indicates soybean oil; see table 1 for other diet components. (For details on experiments 4 and 9, see tables 3 and 4, footnote 1. Experiment 3 utilized duplicate groups of 10 Broad Breasted Bronze male turkey poults. Experimental period 7 to 28 days; initial weight approximately 95 gm.)

The influences of various amino acids and the penicillin-streptomycin combination on the growth depression achieved with diet C containing 35% of ground soybeans is shown in table 4. Data obtained using the control diet (A) indicate that the addition of DL-methionine resulted in excellent stimulation in growth rate; however, further supplementation with tyrosine, valine and threonine did not improve growth. The addition of the antibiotics resulted in a 13% growth response over the basal diet and a similar response over the diet containing 0.3% of DL-methionine.

With the diet containing 35% of ground soybeans, methionine supplementation resulted in a 52% growth response and again the additional amino acids did not produce growth stimulation. The addition of antibiotics produced a larger growth response (28%) over that with the basal than obtained in the presence of methionine (9%). The response with combined supplementation (65%) was greater than the corresponding response (24%) with the soybean meal diet. Growth with the supplemented raw soybean diet was not significantly poorer than that with the unsupplemented soybean meal diet.

[7] See footnote 4.

TABLE 3

Effect of antibiotics on growth of turkeys fed ground raw soybeans (exp. 4)[1]

Diet and supplementation	Weight gain	MRT[2]	Response	Efficiency
	gm		%	
Soybean meal (diet A)				
None	402.6	a	—	0.57
Antibiotics[3]	475.2	b	18	0.61
20% Ground soybeans (diet B)				
None	275.3	c	—	0.43
Antibiotics[3]	444.1	ab	61	0.51
68% Ground soybeans (diet D)				
None	90.3	d	—	0.23
Antibiotics[3]	139.6	d	55	0.24

[1] Treatment values are average of duplicate groups of Broad Breasted Bronze poults (4♂, 4♀ per group). Experimental period was from 7 to 28 days of age; initial weight approximately 87 gm.

[2] For MRT (Duncan's multiple range test), see Experimental section in text.

[3] Antibiotics: 0.1% of procaine penicillin G and 0.1% of streptomycin sulfate.

TABLE 4

Effect of amino acids and antibiotics on raw soybean growth depression (exp. 9)[1]

	Diet and supplementation	Weight gain	MRT[2]	Response		Efficiency
		gm		%	%	
Soybean meal (diet A)						
1	None	408	a	—	—	0.63
2	0.3% DL-methionine	474	bc	16.2	16.2	0.66
3	0.6% DL-methionine	501	bc	22.8		0.67
4	Four amino acids[3]	491	bc	20.4		0.68
5	Antibiotics[4]	461	b		13.0	0.64
6	As 2 + 5	505	c		23.9	0.71
35% Ground soybeans (diet C)						
7	None	223	d	—	—	0.45
8	0.3% DL-methionine	285	e	28.1		0.47
9	0.6% DL-methionine	338	f	51.9	51.9	0.52
10	Four amino acids[3]	345	f	55.0		0.54
11	Antibiotics[4]	285	e		28.1	0.47
12	As 9 + 11	368	fa		64.9	0.52

[1] Treatment values are averages of triplicate groups of Broad Breasted White turkey poults. Each group contained 4 male and 4 female turkeys. Experimental period was from 7 to 28 days; starting weight approximately 95 gm.

[2] See table 3, footnote 2.

[3] Four amino acids are 0.6% of DL-methionine, 0.3% of L-tyrosine, 0.4% of DL-valine, and 0.3% of DL-threonine.

[4] Antibiotics are 0.1% of procaine penicillin G and 0.1% of streptomycin sulfate.

TABLE 5

Effect of enzyme sources and bacitracin on raw soybean growth depression (exp. 9)[1]

Diet and supplementation[2]	Weight gain	MRT[3]	Response	Efficiency
	gm		%	
Soybean meal (diet A)				
None	408	a	—	0.61
0.25% Enzyme C-2	423	a	3.8	0.63
0.25% Enzyme D-7	415	a	1.6	0.62
0.25% Enzyme B-4	424	a	3.9	0.62
0.0165% Bacitracin	435	a	6.7	0.64
35% Ground soybeans (diet C)				
None	223	b	—	0.45
0.25% Enzyme C-2	218	b	−2.3	0.44
0.25% Enzyme D-7	236	b	6.1	0.44
0.25% Enzyme B-4	251	b	12.9	0.46
0.0165% Bacitracin	262	b	17.7	0.46

[1] See table 4, footnote 1.

[2] Enzyme supplements are products of Commercial Solvents Corporation, Terre Haute, Indiana, and were described as follows: Enzyme C-2, High-peptidase activity, supplies diet with 0.0046% bacitracin; enzyme D-7, high-proteinase, low-peptidase activity, supplies diet with 0.0082% bacitracin; enzyme B-4, high-proteinase and peptidase activity, supplies diet with 0.0168% bacitracin.

[3] See table 3, footnote 2.

The possibility that sources of dietary enzymes might have some effectiveness in overcoming the growth depression of raw soybeans was tested in a separate comparison (table 5). Although the enzyme supplements were particularly suited to protein-splitting activity, they also contained some bacitracin. Therefore, a further group was included to study the effect of bacitracin, per se. The use of bacitracin alone gave a somewhat greater response with the raw soybean-containing diet than with the control diet (18% vs. 7%). The enzyme supplements exhibited no activity beyond that of their respective bacitracin contents.

The effect of added dietary zinc on the performance of turkeys fed ground raw

TABLE 6

Effect of supplementary zinc on turkey growth using various levels of raw ground soybeans (exp. 9)[1]

Diet and supplementation	Weight gain	MRT[2]	Response		Efficiency
	gm		%	%	
Soybean meal (diet A)					
No supplemental zinc	398.0	a	—		0.61
40 ppm Added zinc	407.9	a	2.4	—	0.63
35% Ground soybeans (diet C)					
No supplemental zinc	203.7	b	—		0.40
40 ppm Added zinc	222.8	b	9.4		0.45
68% Ground soybeans (diet D)					
No supplemental zinc	84.2	e	—		0.21
40 ppm Added zinc	85.4	e	1.4		0.21
Heated ground soybeans (diet D)					
Autoclaved[3] (40 ppm zinc)	327.2	d		−19.8	0.56
Commercial[3] (40 ppm zinc)	341.3	d		−16.3	0.55

[1] See table 4, footnote 1.
[2] See table 3, footnote 2.
[3] See Experimental section in text for details of preparation.

soybeans is presented in table 6. Although the addition of zinc did not yield a significant response, the 9.4% response with 35% of ground soybeans may have biological importance. In either case, the response was minor compared with the magnitude of the growth depression.

Also included in table 6 is the result where poults received two heated ground soybean products. Growth rate was greatly improved in the turkeys receiving the heated products compared with that of those fed raw soybean-containing diets; however, it was still significantly poorer than in the control-containing soybean meal and oil.

DISCUSSION

The control diet in this study was a typical corn-soybean meal type of turkey starting ration, except that it contained 12% added soybean oil. Similar diets containing 15% of animal fat require at least 0.2% of supplementary methionine for maximal growth (Waibel, '59). That methionine is the critical limiting amino acid even with the use of properly processed soybean meal would explain why physiologic inefficiency, e.g., inhibition of intestinal proteolysis (Alumot and Nitsan, '61) and loss of pancreatic secretions (Lyman and Lepkovsky, '60) aceentuates the methionine deficiency.

The observation that a given level of supplementary methionine was able to produce a greater percentage growth response with the raw soybean diet than with the control diet would seem to be a reflection of the law of diminishing returns (log-dose response) relationship (Almquist, '53). If methionine were the only limiting nutrient, then adding sufficient methionine should completely correct the growth depression. Sufficient methionine levels were not included in experiment 9 to determine this point.

It is likely that if raw soybeans contribute a small portion of the total protein intake, then methionine might completely reverse the growth depression, on the basis that no other amino acids were deficient. It is apparent that other amino acids are also limiting when substantial amounts of raw soybeans are fed (Almquist and Merritt, '53; Fisher and Johnson, '58; Borchers, '59). The amino acids suggested by Borchers ('59) as being limiting for rats were used in experiment 9 (table 4), but did not exhibit any growth-stimulating effect for the turkey. This apparent inconsistency may be explained by species requirement differences or dietary amino acid balance at a given protein level.

The efficacy of antibiotics in counteracting the growth-retarding effect of raw soybeans seemed to be related to the dose of raw soybeans. At the 20% level of raw soybeans, the antibiotics essentially reversed the growth depression. At higher raw soybean levels a lesser portion of the

growth depression was counteracted. That antibiotics counteract the growth depression due to the raw soybean defect(s) in the turkey confirms the findings of Borchers et al. ('57) and Borchers ('58) with the rat and Braham et al. ('59) with the rat and chick. It would appear from the present study that the antibiotics are more effective in reversing the growth depression when lower levels of raw soybean are present. With a more complex deficiency, the antibiotics are limited in their "sparing" ability.

The growth response to antibiotics in experiment 9 (table 4) using control diet A with or without methionine and diet C (35% raw soybeans) with methionine ranged from 6 to 13%. However, the antibiotics produced a 28% response with diet C in the absence of methionine supplementation. This observation suggests a methionine-sparing action by the antibiotics, in confirmation of the Borchers finding with rats fed the 40% level of raw soybean meal.

An excellent discussion of the manner in which antibiotics act in counteracting the raw soybean defect was given recently by Braham et al. ('59). They stated that it was "unlikely that the antibiotics were sparing some limiting nutrient;" however, in the present investigation the antibiotics appeared to "spare" the methionine requirement where methionine was deficient in a raw-soybean containing diet. As these authors suggested, the antibiotics might affect the digestibility or absorption of the protein of the raw meal, presumably as a consequence of action on the intestinal flora. A further possibility requiring investigation involves systemic activity of the antibiotics as shown by Hartsook et al. ('59). They reported that antibiotics inhibited kidney xanthine dehydrogenase activity in the chick, and suggested activity of the antibiotics through increased nitrogen retention. In a similar manner, the possibility exists that antibiotics might inhibit pancreatic enzyme formation and critical loss of pancreatic secretions (Lyman, '60).

Almquist and Merritt ('52) noted in chicks that the growth inhibition associated with varying proportions of raw-to-cooked soybeans was almost fully developed when only ¼ of the protein in 20 or 30% protein diets was supplied in the raw form. This was interpreted to indicate the presence of a trypsin inhibitor, the activity of which was essentially complete at this level of raw soybeans. The growth inhibition in this study with the turkey does not confirm that of Almquist with chicks. The factor(s) responsible for the growth depression was directly related to growth rate over the entire range of raw soybean administration in our study.

Since Kratzer et al. ('59) observed that the biological availability of zinc was low in diets containing isolated soybean protein, it seemed that growth depression with the raw soybean might be the result, at least in part, of a zinc deficiency. As shown in table 6, the response to zinc supplementation (40 ppm) was not of sufficient magnitude to result in statistical significance. In previous experiments,[8] the addition of 100 ppm of zinc to the diet already containing 20 ppm of zinc also did not reverse the growth depression observed.

The lack of effectiveness of added dietary enzyme sources in improving raw soybean utilization adds circumstantial support to the observation of Lyman and Lepkovsky ('60) that enzyme activity in the gut is not the limiting factor when raw soybeans are fed.

Renner and Hill ('60) showed that the low nutritive value of raw soybeans for the chick is due in part to poor digestion or absorption of fat, or both, and that flaking of the beans increases the fat absorbability. In the present study (table 2) efficiency of feed utilization was better when flaked soybeans were used.

SUMMARY

The isonitrogenous-isocaloric inclusion of raw ground soybeans into a corn-soybean meal diet produced a depression in growth in young turkeys which was linearly related to dose when plotted semilogarithmically. The effect of antibiotics in counteracting the growth-retarding effect of raw soybeans was related to the dose of raw soybeans. When the diet contained 68% of ground raw soybeans, the antibiotics (0.1% of procaine penicillin

[8] See footnote 4.

G and 0.1% of streptomycin sulfate) stimulated growth somewhat, but were largely ineffective in reversing the depression. Supplementing diets containing 35% of ground raw soybeans with the antibiotics, methionine, or both, resulted in greater growth responses than for respective controls receiving processed soybean meal and soybean oil, thus indicating activity in helping reverse the raw soybean defect. When the diet contained 20% of ground raw soybeans, the antibiotics essentially reversed the raw soybean growth depression. Zinc supplementation was not beneficial at either the 68% or the 35% level of raw soybean meal; likewise, three dietary enzyme products also proved ineffective at the latter level.

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Net Protein Values for the Growing Chicken as Determined by Carcass Analysis: Exploration of the Method[1]

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The nutritive evaluation of proteins in avian nutrition has not received the careful attention it deserves because of the difficulty in carrying out classical balance studies in a species in which urine and feces are voided jointly. The few such studies that are reported have involved either surgical modification of the bird (Ariyoshi, '57) or a chemical separation of urine and feces from a mixed collection of total excreta (St. John et al., '32; Wetterau, '54; Göbel, '60). Aside from the fact that both of these approaches are based on assumptions that cannot be checked experimentally, these methods are not easily adapted to routine and large scale testing of commonly available protein sources.

Despite the dearth of information on protein quality for growing chickens by nitrogen balance techniques, little such information is available from the less cumbersome growth method. Hinners and Scott ('60) recently examined the growth method for determining protein quality in the chicken but made no attempt to introduce changes that would overcome the long-standing criticism of this method (Mitchell, '44). Some of the shortcomings of the growth method, namely (a) that the maintenance requirements of the test animals is not taken into consideration; (b) the variation of results with different food intakes, and (c) the assumption that gain in body weight is indicative of the protein tissue laid down, have recently been examined in the rat and appropriate corrections proposed (Bender and Doell, '57). These consist of feeding a nitrogen-free diet to a group of control animals. The difference between the weights of this group and that of the test animals is used instead of the weight gain of the test animals only. This procedure makes allowances for the maintenance requirement and also permits the evaluation of poor quality proteins which do not promote growth.

The carcass retention method as developed by Bender and Miller ('53a) with the growing rat offers an opportunity to obtain net protein values in the chicken without recourse to surgery or chemical separation of excreta. The usefulness and close correlation of the carcass retention method with the classical balance technique has been confirmed in the rat by Forbes and Yohe ('55), and recently the application of the method to the chicken has been suggested in a limited study by De Muelenaere et al. ('60).

The present study was concerned with the development of a routine protein evaluation procedure involving carcass nitrogen retention for the determination of net protein values in the growing chicken. At the same time, the modified growth assay of Bender and Doell ('57) has also been examined and tested.

EXPERIMENTAL

Male crossbred (New Hampshire males × Columbian females) chicks were used throughout these trials. Day-old chicks were supplied with a standard ration for a one-week period after which time they were assigned by weight to their respective treatment diets. The average body weight

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for the birds at the start of the experimental periods was 80 gm. Four replicates of 5 birds were used for each of the test rations which included also a nitrogen-free regimen. All rations were analyzed for nitrogen by semimicro Kjeldahl analysis. The experimental rations were fed for a two-week period. The composition of the rations is shown in table 1.

Twelve hours prior to the termination of the experiment, feed was removed. The birds were weighed, then killed with chloroform and dried to constant weight in a forced-air oven at 85°C. Moisture values were obtained by difference between the final fresh weight of the birds and their dried weight. The dried carcasses were then ground, mixed well and samples taken for nitrogen determination; fat was determined by extraction with chloroform-methanol (2:1), evaporation of the solvents and re-extraction with petroleum ether. The residue left after evaporation of the petroleum ether was considered to be fat. All chemical determinations were carried out in duplicate or triplicate.

Definition of terms. Net protein value (NPV) is defined by Bender's equation as follows:

$$NPV = \frac{B_f - B_k + I_k}{I_f}$$

where B_f and I_f denote carcass nitrogen and nitrogen intake of animals fed the test diet, respectively, and B_k and I_k equal carcass nitrogen and nitrogen intake with the nitrogen-free control diet.

Protein retention efficiency (PRE) is defined by the following equation:

$$PRE = \frac{G_f - G_k}{P_f} \times 18.0$$

where G_f and G_k denote gain or loss in body weight with the test and nitrogen-free rations, respectively; P_f equals the protein intake with the test ration, and 18.0 is the determined (trial 3) average percentage carcass protein (wet-weight) of the birds reared under the specific conditions of this study.

TABLE 1

Composition of experimental rations

	%
Purified diet (trials 1–4)	
Corn starch	28.00
Dextrin	5.00
Mineral mix[1]	5.90
Fiber[2]	3.00
Corn oil	3.00
Choline chloride	0.20
Vitamins[3]	0.25
Glucose	to 100
	%
Practical diet (trial 1)	
Corn meal[4]	86.2
Soybean meal (50% protein)	10.0
Dicalcium phosphate	2.0
Limestone with trace minerals[5]	1.0
Sodium chloride	0.5
Vitamins[3]	0.2
Choline chloride	0.1
	100.0

[1] As percentage of total mineral mix: $Ca_3(PO_4)_2$, 17.2; KH_2PO_4, 21.3; NaCl, 16.2; $CaCO_3$, 38.5; Fe gluconate, 1.05; $MgSO_4$, 5.06; $MnSO_4 \cdot H_2O$, 0.4; KI, 0.02; $CuSO_4$ anhyd., 0.025; $ZnCO_3$, 0.40; $Na_2MoO_4 \cdot 2H_2O$, 0.02.

[2] The 3% fiber was omitted from rations containing soybean, peanut and sesame oil meal (trials 3 and 4) since these ingredients contain a considerable amount of bulk.

[3] In milligrams per kilogram of diet: thiamine·HCl, 25; riboflavin, 16; Ca pantothenate, 20; vitamin B_{12}, 0.02; pyridoxine·HCl, 6; biotin, 0.6; folic acid, 4; 2-methyl naphthoquinone, 5; ascorbic acid, 250; niacin, 150; per kilogram of diet: vitamin A, 10,000 IU; vitamin D_3, 600 IU; and α-tocopheryl succinate, 5 IU.

[4] The levels of corn and soybean meal listed represent the composition of the 13% protein ration; in the 21% protein ration 66.2% of corn and 30.0% of soybean meal were used.

[5] Mico Concentrate, Limestone Corporation of America, Newton, New Jersey.

RESULTS

Trial 1. In the first trial, carcass composition of growing chickens was compared after feeding either a ration composed of natural ingredients or a purified ration containing isolated soybean protein[2] and glucose. Two levels of protein were tested in each ration to compare carcass composition and in particular the water:nitrogen ratio (W:N). This ratio, according to Bender and Miller ('53b), remains constant in the rat under varied feeding conditions and can therefore be used to estimate carcass nitrogen from the moisture content. In trial 1 all analyses were carried out on individual birds so that a total of 20 animals were analyzed for each dietary treatment.

The data in table 2 indicate that despite substantial and significant changes

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

TABLE 2

Body weights, carcass composition and net protein values of chickens fed practical or purified rations[1]

Measurement	Nitrogen-free diet	Purified diet		Practical diet	
		13% protein[2]	26% protein[2]	13% protein[3]	21% protein[3]
3-Week weights, gm	55 ± 1[4]	225 ± 4	274 ± 5	173 ± 6	262 ± 5
Carcass:					
Moisture, %	66.1 ± 0.5	65.5 ± 0.1	69.9 ± 0.2	65.1 ± 0.3	70.2 ± 0.2
Fat (% dry-weight)	34.4 ± 1.2	37.5 ± 1.1	25.6 ± 1.2	38.4 ± 0.7	21.9 ± 0.7
Nitrogen (% dry-weight)	7.71 ± 0.15	7.83 ± 0.13	9.72 ± 0.19	7.57 ± 0.05	9.80 ± 0.04
Water:nitrogen ratio	25.4 ± 0.3	24.7 ± 0.3	23.6 ± 0.2	24.7 ± 0.2	24.1 ± 0.2
Net protein values		66.9 ± 1.3	47.9 ± 0.5	50.4 ± 0.5	52.3 ± 0.9

[1] Week-old male chickens were fed the experimental rations for two weeks; average starting weight was 80 gm. The three-week weights as well as the moisture values are averaged for quadriplicate groups of 5 birds; values for fat and nitrogen are averages of duplicate or triplicate determinations on quadriplicate groups of 5 birds (20 birds)/treatment.

[2] ADM Assay Protein C-1, an isolated soybean protein (Archer-Daniels-Midland Company, Cincinnati), supplemented with 0.2 and 0.4% DL-methionine at the 13 and 26% protein levels, respectively.

[3] At the 13% protein level approximately 40% of the protein was supplied by corn meal and 60% from soybean oil meal; at the 21% protein level 70% of the protein was from soybean oil meal with corn meal supplying only approximately 30%.

[4] Mean value with its standard error.

in moisture, fat and nitrogen content of the carcass, the W:N ratio remains nevertheless constant. Indeed, variance analysis shows no significant difference ($P > 0.05$) among the W:N ratios obtained with the various rations. The coefficients of variation for individual W:N ratios with the 5 different rations were of the order of 4%, whereas for the carcass nitrogen values the coefficients of variation were 8%.

Also shown in table 2 are the net protein values for the different rations. The rations containing isolated soybean protein at the 13% level showed a much higher net protein value than the combined proteins from corn and soybean supplied at the same protein level in the practical ingredient ration. This is not unexpected in view of the considerable proportion of corn protein in the practical ration which reduces the effectiveness of the soybean protein. In one of the later experiments (trial 3) soybean meal protein was observed to have a higher net protein value than the combination of corn and soybean protein. This is also substantiated by the fact that the net protein value improved slightly instead of decreasing, as total protein was increased from 13 to 21% as noted with the isolated soybean protein. This improvement is undoubtedly due to the relatively greater proportion of soybean meal protein to corn protein in the 21 as compared with the 13% protein ration (see footnote 2, table 2).

Trial 2. The purpose of this study was to compare NPV and carcass composition over 5 levels of dietary protein. In this experiment only the purified, isolated-soybean protein ration was used with a view towards using this protein as a reference standard for future studies. This protein, properly supplemented with methionine (table 3, footnote 1) was fed at levels ranging from 12 to 27%. Because of the low variability between birds receiving the same treatment, in carcass composition, particularly for the W:N ratios (trial 1), pooled analyses were carried out on each of the 4 replicates of 5 birds in trial 2.

In table 3 are given the carcass composition analyses as well as the W:N ratios and the net protein values. Again, with differing fat and nitrogen percent-

TABLE 3

Body weights, carcass composition and net protein values of chickens fed increasing levels of isolated soybean protein[1]

Measurements	Rations					
	Nitrogen-free	Protein[2] (%)				
		12	16	18	21	27
3-Week weights, gm	61 ± 1[3]	232 ± 6	252 ± 7	265 ± 6	285 ± 4	280 ± 6
Carcass:						
Moisture, %	68.1 ± 0.3	65.4 ± 0.5	66.9 ± 0.4	70.0 ± 0.6	70.1 ± 0.5	71.5 ± 0.3
Fat, % dry-weight	31.2 ± 0.4	34.6 ± 0.6	29.4 ± 0.7	25.5 ± 0.7	23.0 ± 1.0	21.8 ± 0.7
Nitrogen, % dry-weight	8.06 ± 0.03	7.63 ± 0.13	8.37 ± 0.05	9.00 ± 0.04	9.46 ± 0.05	10.06 ± 0.05
Water:nitrogen ratio	26.5 ± 0.4	24.8 ± 0.5	24.2 ± 0.3	25.4 ± 0.5	24.9 ± 0.6	25.0 ± 0.2
Net protein values		68.6 ± 0.5	62.2 ± 1.7	58.3 ± 1.7	54.1 ± 0.7	45.5 ± 0.9

[1] Week-old male chickens were fed the experimental rations for two weeks; average starting weight was 80 gm. The three-week weights are averages of quadriplicate groups of 5 birds; values for moisture, fat and nitrogen are averages of the 4 replicates representing the pooled samples of the 5 birds in each replicate. Fat and nitrogen determinations were carried out in duplicate or triplicate.

[2] ADM Assay Protein C-1, an isolated soybean protein (Archer-Daniels-Midland Company, Cincinnati). The precise protein (N × 6.25) analyses of the rations were: 11.8, 15.8, 18.2, 21.4 and 26.6%. DL-Methionine was added to these rations in the following amounts: 0.17, 0.21, 0.25, 0.29 and 0.32% of the diet.

[3] Mean with its standard error.

ages of the carcass the W:N ratios were remarkably constant both within (note small standard errors) and among treatment groups. In figure 1 are shown the net protein values plotted against the level of dietary protein. The linear relationship was highly significant ($P < 0.001$) and showed that the isolated soybean protein supplemented with methionine is a reasonably good protein source for the growing chicken.

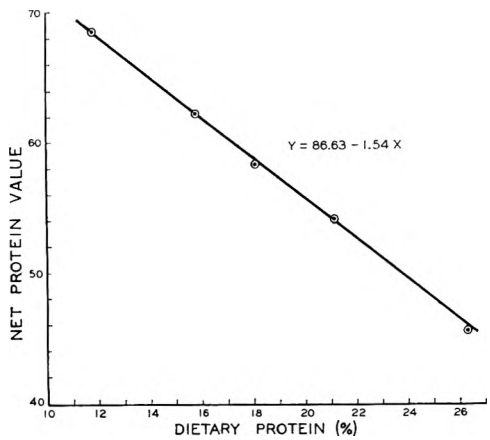

Fig. 1 Regression line of dietary protein level on net protein values. Points shown represent averages of actual observations.

Trial 3. This experiment was based on the experience gained in the first two trials with respect to the development of a practical assay procedure for the determination of net protein values. Such values were determined for 5 proteins that are of interest in avian nutrition. These proteins were either given alone or supplemented with those amino acids in which they are known to be deficient. The test protein was supplied at a level of approximately 13%. Based upon the experience of trials 1 and 2, the 5 carcasses of only one of the 4 replicates for each treatment were pooled for nitrogen analyses, whereas the other three replicates were dried only, and nitrogen estimated from the W:N ratios. These ratios were determined from the average carcass nitrogen values obtained by analysis from the one replicate of all treatments. The data obtained are shown in tables 4 and 5. The W:N ratios from this trial, based on determined carcass nitrogen from one replicate, are in line with the ratios previously determined (23.1 versus 24.7). There was also good agreement between the carcass nitrogen determined in trials 1 and 2 from the single replicate and the calculated carcass

TABLE 4

Carcass moisture and nitrogen content of chickens fed different dietary proteins with and without amino acid supplementation[1]

Protein supplement	Carcass composition			
	Moisture	Determined N	Calculated N	Water/ ntirogen
	%	*% dry weight*	*% dry weight*	
Isolated soybean protein[2] + 0.17% DL-methionine	65.3 ± 0.4[3]	7.91	8.22 ± 0.15	22.9
Soybean oil meal[4]	66.5 ± 0.7	8.37	8.71 ± 0.38	22.9
Soybean oil meal + 0.17% DL-methionine	64.9 ± 0.7	8.17	7.89 ± 0.29	23.5
Sesame meal[5]	66.2 ± 0.5	9.10	8.35 ± 0.24	22.3
Sesame meal + 0.5% L-lysine·HCl	65.9 ± 0.6	8.35	8.31 ± 0.33	23.6
Peanut meal[6]	67.6 ± 0.6	9.22	8.78 ± 0.17	24.5
Peanut meal + 0.17% DL-methionine	67.6 ± 0.4	8.95	9.11 ± 0.17	22.7
Peanut meal + 0.17% DL-methionine + 0.5% L-lysine·HCl	66.4 ± 0.2	8.18	8.63 ± 0.06	23.7
Whole egg[7]	64.3 ± 0.2	8.10	7.73 ± 0.07	22.6
Nitrogen-free	66.4 ± 1.0	8.52	8.98 ± 0.28	22.2

[1] Week-old male chickens were fed the experimental rations for two weeks; average starting weight was 80 gm. The moisture determinations represent averages of 4 pooled replicates of 5 birds/treatment. The determined nitrogen values represent averages of triplicate determinations from a pooled sample of 5 birds from one of the 4 replicates on each treatment; the calculated nitrogen values were obtained from the moisture values in column 2 and the average of the W:N values in column 5 (23.1). The W:N values were calculated from the data in columns 2 and 3.

[2] ADM Assay Protein C-1, Archer-Daniels-Midland Company, Cincinnati. The diet contained 12.0% (N × 6.25) by analysis.

[3] Mean with its standard error.

[4] A solvent-extracted toasted commercial meal; the soybean oil meal diets contained respectively 12.9 and 13.1% (N × 6.25) by analysis.

[5] A hydraulically pressed feed grade meal; the sesame diets contained, respectively, 11.6 and 12.8% (N × 6.25) by analysis.

[6] A commercial feed grade meal; the peanut meal diets contained, respectively, 14.1, 13.3 and 14.1% (N × 6.25) by analysis.

[7] A low-temperature solvent extracted and dried preparation containing 80% protein; this diet contained 11.5% (N × 6.25) by analysis.

nitrogen content of the other replicates based upon the W:N ratios.

With respect to the net protein values for the sources tested in this trial, excellent agreement was noted between the net protein values obtained for the isolated soybean protein in all three trials. The particular source of whole egg protein used (a low-temperature solvent-extracted meal) was of comparatively poor quality and no better than either the isolated soybean protein or the proteins from intact soybean or sesame meal. The peanut meal protein was of very poor quality, even when supplemented.

In table 5 are also shown the protein retention efficiencies (PRE) as defined by Bender and Doell ('57). These generally agree well with the net protein values, suggesting that Bender's modification of the growth method, namely the inclusion of a nitrogen-free group to account for maintenance and changes in food intake, will provide a reliable estimate for protein quality in the growing chicken. To further test the usefulness of the growth method (PRE) a duplicate experiment (trial 4) was carried out with the same design as trial 3. In this experiment no carcass analyses were carried out; only growth data were obtained, and the PRE values were calculated using the average carcass protein content of trial 3 (18.0%). Comparison of these values with those obtained in trial 3 (columns 5 and 7, table 5), as well as with the net protein values of trial 3, point to the usefulness of this method, particularly since the PRE values of trial 4 were determined with an independent group of animals.

DISCUSSION

Although it is difficult to compare in the chicken the carcass retention method with

TABLE 5

Body weights, net protein values and protein retention efficiencies of chickens receiving different dietary proteins with and without amino acid supplementation[1]

Protein supplement[2]	Trial 3				Trial 4	
	3-Week weights	NPV[3] determined	Calculated	PRE[4]	3-Week weights	PRE[4]
	gm				*gm*	
Isolated soybean protein + 0.17% DL-methionine	192 ± 5[5]	69.1	70.6	68.9	207 ± 5	67.3 ± 1.1
Soybean oil meal	155 ± 10	55.1	57.6	51.7	161 ± 10	46.0 ± 2.9
Soybean oil meal + 0.17% DL-methionine	208 ± 7	68.8	63.4	65.5	214 ± 4	62.0 ± 0.9
Sesame meal	98 ± 3	56.1	48.1	44.3	99 ± 4	43.2 ± 0.7
Sesame meal + 0.5% L-lysine·HCl	198 ± 8	62.7	63.4	62.6	223 ± 5	63.6 ± 1.7
Peanut meal	96 ± 3	39.4	39.2	34.9	92 ± 3	31.5 ± 0.5
Peanut meal + 0.17% DL-methionine	92 ± 2	41.7	39.4	36.7	93 ± 3	37.8 ± 0.8
Peanut meal + 0.17% DL-methionine + 0.5% L-lysine·HCl	130 ± 6	46.2	45.3	45.7	128 ± 8	41.4 ± 1.5
Whole egg	160 + 10	66.0	65.6	63.4	182 ± 6	65.2 ± 1.6
Nitrogen-free	58 ± 1	—	—	—	59 ± 2	—

[1] Week-old male chickens were fed the experimental rations for two weeks; average starting weight was 80 gm. The three-week body weights represent the average of 4 replicates of 5 birds per treatment.

[2] For details see footnotes in table 4.

[3] Net protein values; see definition in text. The determined values were obtained using the carcass nitrogen values from one replicate of 5 birds (column 3, table 4), while the calculated values were derived using the average W:N value of column 5, table 4 (23.1).

[4] Protein retention efficiency; see definition in text.

[5] Mean with its standard error.

the balance technique, as Forbes and Yohe ('55) did in their studies with rats, it appears, nevertheless, that the carcass retention method is suitable and can be used with reliability for the growing chicken. The reliability of the method is emphasized by the high degree of correlation ($r > 0.9$) between the net protein values and the protein retention efficiencies as determined in separate experiments (trials 3 and 4, table 5). The usefulness of the method is accentuated by the relative constancy of the W:N ratios both within and between experiments, obviating the need for (a) analyses on individual birds, and (b) carcass nitrogen determinations. The following assay procedure is therefore proposed.

Week-old chicks of one sex are separated into groups of 5, and 4 replicates assigned to each test diet, including a nitrogen-free regimen. The birds are given the experimental ration containing 13% of protein for two-weeks, at the end of which time they are starved for 12 hours, and killed. Moisture determinations are carried out on all groups, and carcass nitrogen is determined on one of the 4 replicates per treatment. Water:nitrogen ratios are calculated for the one replicate and carcass nitrogen values estimated for the three remaining replicates from the average of the W:N ratios. NPV or PRE or both can be calculated from the equations given previously. If repeated assays are carried out, it will not be necessary to carry out N determinations on any replicate; instead, the W:N ratios and the conversion factor for calculating PRE derived from prior determinations will suffice.

The choice of the test protein level of approximately 13% of the ration in these studies deserves comment. This level seemed reasonable since it permits suboptimal growth, yet is sufficiently high to afford the opportunity to distinguish between the amino acid requirements for maintenance and growth. In our opinion too low a protein level can give a distorted picture of protein quality for growth in view of the differences in the maintenance versus the growth requirements for some of the essential amino acids. Such differences are of particular importance in the case of the two amino acids most likely to be deficient in the common protein sources available for the feeding of chickens, namely methionine and lysine (Fisher et al., '60). Thus, if too low a protein level were fed the proportion of test protein available for growth might be so small that its true growth promoting value might be effectively masked by the disproportionate utilization of certain amino acids for maintenance purposes. De Muelenaere et al. ('60) used a protein level as low as 7% in their study which provides little excess above the protein maintenance requirement of the chicken. This appears to have led these workers to conclude that the chicken differed from the rat since they found a factor of 13 for the calculation of the PRE values compared with a value of 16 obtained by Bender in the rat; as indicated previously, in the present study a value of 18 was determined.[3] It appears more logical that these differences are due not to a species difference but rather to a difference in carcass nitrogen content resulting from differing nitrogen intakes. Thus, with De Muelenaere's ration, containing only 7% of protein, a factor of 13 was obtained, whereas Bender, using a 10% protein ration in the rat obtained a value of 16, and with a 13% protein ration we obtained a value of 18. The effect of dietary protein on carcass nitrogen is well illustrated in table 3.

The linear decrease in net protein value with increasing level of dietary protein agrees with the observations made by Forbes et al. ('58) on a series of proteins in the growing rat. In fact, the slope and intercept for the isolated soybean data in the chicken (fig. 1) correlated closely with those for the rat for a protein of similar biological value.

The net protein values obtained for the test proteins indicate an unexpectedly low value for egg, presumably the result of processing. The isolated and intact soybean proteins as well as sesame protein, when properly supplemented with the deficient amino acids, were good sources of protein for the growing chicken. The very low value of peanut protein (in both trials

[3] Theoretically the correct factor ought to equal the per cent protein of the gain in body weight. In practice we used the percentage of protein in the total carcass.

3 and 4) even when supplemented with lysine and methionine confirms the results of De Muelenaere et al. ('60). This protein needs further study since, when supplied at a higher level, it will support good growth similar to that of soybean meal (Fisher et al., '60). Finally, the inconsistencies between weight gain and NPV, as illustrated in table 5, point up the advantage of the N retention method which actually accounts for the protein consumed and digested. For example, the NPV for both unsupplemented soybean and sesame meal are the same, whereas the three-week weights differ by more than 50%.

SUMMARY

A nitrogen retention method for use in the growing chicken has been outlined, based upon one originally described by Bender and Miller ('53) for the growing rat. The method involves the feeding of 13% protein rations together with a nitrogen-free ration to quadruplicate groups of 5 birds. It was shown that the carcass water:nitrogen ratios were remarkably constant despite considerable variations in nitrogen and fat content of the carcass on various diets. The net protein value of isolated soybean protein decreased linearly with increasing level of dietary protein over the range of 13 to 27%. Five protein sources were studied of which isolated soybean and soybean meal protein were observed to be of comparable value. A commercial sample of defatted egg powder was noted to be below expectation. Peanut meal protein was very poorly utilized even when supplemented with methionine and lysine.

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Studies on the Combined and Relative Influence of Dietary Protein and Riboflavin in Flavoprotein Enzymes

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Xanthine oxidase (XO) and D-amino acid oxidase (DAO) are two important flavoprotein enzymes that contain flavin adenine dinucleotide (FAD) as their coenzyme. The activities of these two enzymes in liver are influenced by riboflavin and protein intake.

Liver xanthine oxidase activity decreases in animals suffering partial or complete protein deficiency (Mac Quarrie and Venosa, '45; Lang, '47; Westerfeld and Richert, '49, '50; Miller, '50; Litwack et al., '50; Wainio et al., '53; Bro-Rasmussen, '58). Liver D-amino acid oxidase activity also decreases in animals fed either a low-protein or protein-free diet (Axelrod et al., '42; Lang, '47; Seifter et al., '48; Benditt et al., '49; Vanderlinde and Westerfeld, '50; Wainio et al., '53; Srinivasan and Patwardhan, '55), although according to Hawkins ('52) the liver D-amino acid oxidase activity is more specifically dependent upon riboflavin.

Decker and Byerrum ('54) have shown that both xanthine oxidase and D-amino acid oxidase activities in rat liver are influenced by dietary riboflavin and occur least in the livers of rats fed a diet unsupplemented with riboflavin.

The scope of the present studies was to determine how much the two flavoprotein enzymes were affected in a combined deficiency of protein and riboflavin; also to investigate to what extent the two influencing factors, i.e., dietary protein and riboflavin, affected these flavoprotein enzymes, with a view to assessing their relative influence.

METHODS AND MATERIALS

Albino rats obtained from Nutritional Research Laboratories, Coonoor, Nilgiris, India, were used. They weighed 70 ±10 gm at the beginning of the experiment. They were grouped into 8 groups of 4 rats each. Groups 1, 2, 3, and 4 received 5, 18, 40, and 60%, respectively, of devitaminized casein (by repeated washing with alcohol) in the diet without riboflavin supplement. Groups 5, 6, 7, and 8 also received 5, 18, 40 and 60%, respectively, of devitaminized casein plus 30 μg of riboflavin per rat per day. The percentage composition of the other ingredients of the diet was as follows: arachis oil, 8; salt mixture (Dalgliesh, '52), 4; vitamin mixture not containing riboflavin (Burch et al., '56), 1; cod liver oil, 2; and sucrose to make 100. Five per cent of casein diet without riboflavin was the diet deficient in both protein and riboflavin.

Diets were supplied to the rats at the same quantity, 10 gm per rat per day, but no attempt was made to find out the actual consumption by individual groups of rats. The rats were weighed every week and received the diet for 8 weeks, when they were sacrificed for enzyme estimation.

Xanthine oxidase activity in the liver was estimated colorimetrically by the method of Litwack et al. ('53) using a Lumetron photoelectric colorimeter. D-Amino acid oxidase activity in the liver was estimated by Warburg's manometric method (Umbriet et al., '57) as modified by Decker and Byerrum ('54).

RESULTS AND DISCUSSION

Rats in groups 1 and 5 lost considerable weight even from the second week and had glycosuria. Their blood sugar levels were normal, however.

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Estimation of riboflavin excreted in the urine of the rats by the microbiological assay (AOAC, '55) using *Lactobacillus casei* indicated that more riboflavin was excreted by rats in group 1 than by those in groups 2, 3 and 4 (2.08 µg of riboflavin per mg of creatinine excreted compared with 1.32, 1.84, and 1.1 µg, respectively). Similarly, more riboflavin was excreted by rats in group 5 than by those in groups 6, 7, and 8 (13.5 µg of riboflavin per mg of creatinine excreted, compared with 6.9, 6.2, and 5 µg, respectively). This is in agreement with the observations of many investigators that excretion of riboflavin in urine is greater during protein deficiency (Bro-Rasmussen, '58). Riboflavin was also estimated by microbiological assay in the livers of rats in the various groups. The average total riboflavin content in the livers of the 8 groups of rats expressed in micrograms per gram of wet liver is 11.8, 11.6, 12.3, 13.5, 9.3, 21.3, 22.2, and 22.9, respectively. Rats not receiving riboflavin in the diet showed liver total riboflavin values of 11.6 to 13.5. Of the rats fed with riboflavin in the diet, only group 5 rats showed low total riboflavin content in the liver (9.3 µg per gm of wet liver). This is in agreement with the observation that riboflavin cannot be fully utilized in protein deficiency as the two are mutually interdependent (Czaczkes and Guggenheim, '46; Bro-Rasmussen, '58).

No rat in groups other than 1 and 5 lost weight during the experimental period, although the growth rate of rats receiving supplements of riboflavin was much greater than that of the riboflavin-deficient groups.

Xanthine oxidase activity. Xanthine oxidase activity in liver was least in the combined deficiency of protein and riboflavin (table 1). As xanthine oxidase activity is dependent on both protein and riboflavin, it was affected most in a combined deficiency. Moreover, xanthine oxidase activity of rat liver was much more dependent on dietary protein than riboflavin (table 1). Increase in xanthine oxidase activity due to protein increased with concentration of protein in the diet with a maximal increase with the 60% casein diet. Also, this increase was not greatly affected by the presence or absence of riboflavin in the diet.

D-*Amino acid oxidase activity.* D-Amino acid oxidase activity of rat liver was least in the combined deficiency of protein and riboflavin (table 2). D-Amino acid oxidase activity, being dependent on both dietary protein and riboflavin was affected most in a combined deficiency. Moreover, D-amino acid oxidase activity of rat liver was much more dependent on riboflavin than protein (table 2). However, protein level in the diet had some influence in improving liver D-amino acid oxidase activity and this was more marked in the absence of riboflavin in the diet. Also, when riboflavin was not given, the three levels of protein, 18, 40, and 60%, had more or less the same influence on improving liver D-amino acid oxidase activity. But, when riboflavin was given D-amino acid oxidase activity increased with concentration of protein in the diet and attained a maximum in rats fed the 60% casein diet. This indicates that dietary

TABLE 1

Xanthine oxidase activity of rat liver in micromoles of xanthine oxidized per hour per gram of wet liver

Protein in diet	Xanthine oxidase activity		Change in XO activity due to riboflavin alone	Change in xanthine oxidase activity due to 18, 40 and 60% protein in the diet	
	No dietary riboflavin	With dietary riboflavin		No dietary riboflavin	With dietary riboflavin
%					
5	0.13[1] ± 0.1[2]	0.66 ± 0.2	0.53	—	—
18	6.6 ± 1.2	8.10 ± 0.6	1.50	6.47	7.44
40	9.00 ± 0.8	10.00 ± 0.8	1.00	8.87	9.34
60	13.50 ± 1.5	12.34 ± 1.3	−1.16	13.37	11.68

[1] Average of 4 values.
[2] Mean deviation from average.

TABLE 2

D-Amino acid oxidase activity of rat liver in microliters of oxygen consumed per hour per 100 mg of wet liver (QO_2 alanine)

Protein in diet	D-Amino acid oxidase activity		Change in D-amino acid oxidase due to riboflavin alone	Change in D-amino acid oxidase activity due to 18, 40 and 60% protein in the diet	
	No dietary riboflavin	With dietary riboflavin		No dietary riboflavin	With dietary riboflavin
%					
5	0	89.7[1] ±13.0[2]	89.7	—	—
18	27.3± 6.2	90.60 ± 5.0	63.3	27.3	0.9
40	22.5±14.0	98.95 ±18.3	76.45	22.5	9.25
60	29.7± 5.0	107.3 ±19.0	77.60	29.7	17.60

[1] Average of 4 values.
[2] Mean deviation from average.

protein helps in the utilization of riboflavin (Bro-Rasmussen, '58).

SUMMARY

1. Xanthine oxidase and D-amino acid oxidase activities were determined in the livers of 8 groups of rats with 5, 18, 40 and 60% of devitaminized casein with and without added riboflavin.

2. Both xanthine oxidase and D-amino acid oxidase activities of rat liver were reduced to the minimum in a combined deficiency of dietary protein and riboflavin.

3. Xanthine oxidase activity depended much more upon protein (casein) than upon riboflavin, whereas D-amino acid oxidase activity depended more upon riboflavin than upon protein.

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Metabolic Patterns in Preadolescent Children

VI. VITAMIN B_{12} INTAKE AND URINARY EXCRETION[1]

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Man is dependent upon animal foods for his chief source of vitamin B_{12}. Dietary deficiency of this vitamin has been reported for human beings consuming vegetable diets exclusively (Wokes et al., '55). But there is a general lack of information on the vitamin B_{12} content of diets made up of ordinary foods.

The study reported here was undertaken to obtain information on the vitamin B_{12} content of controlled diets used in a series of studies on metabolic patterns and utilization of selected nutrients by preadolescent girls. Twelve 7- to 9-year-old girls participated in the 1956 study and 11 other girls of like age in 1958. Average daily urinary excretion of vitamin B_{12} was determined for the 11 girls in 1958 when diets of low-protein content were used.

Information on the weighed portions of foods used in the meals for each of the 6 diets, as well as the physical and biochemical characteristics of each girl, are presented in the Southern Cooperative Series Bulletin no. 64 ('59).

PLAN OF STUDIES

The 1956 study consisted of 14 4-day metabolic periods and the 1958 study of 8 6-day periods. A different menu was used on each day within a metabolic period, the menus being repeated in the same order for each period.

All menus were planned to provide the levels of nutrients recommended in the report of the Food and Nutrition Board ('53) except that protein was higher than the recommended amount of 2 gm per kg body weight for 6 of 12 girls in 1956 and lower for all girls in 1958. In 1958, however, the diets provided a sufficient amount of protein to allow for a nitrogen retention of at least 0.3 gm per child per day (James, '60). Hydrolyzed gelatin was used in some diets in 1956 to furnish a portion of the protein (Southern Coop. Series Bull. no. 64, '59).

MATERIALS AND METHODS

Daily at mealtime, weighed aliquots of one-half portion of every food served were placed in a Pyrex jar and frozen. The daily food composites for one metabolic period were blended in a Waring Blendor until the slurry was homogeneous.

All urine for each girl was collected for 24-hour periods, kept under refrigeration during the collection period and held frozen until pooled for each metabolic period.

All samples of pooled urine for each girl and food slurries for the group of girls were kept frozen until the time of analyses.

Vitamin B_{12} was determined by microbiological assays with *Lactobacillus leichmannii* using the AOAC ('55) method with minor modifications (Lichtenstein et al., '61). To insure that the growth promoting activity measured was derived from vitamin B_{12}, checks were carried out with *Ochromonas malhamensis* using a modification (Lichtenstein et al., '61) of Ford's ('53) method.

RESULTS AND DISCUSSION

In table 1 is presented the protein ($N \times 6.25$) content of each of the 6 diets used

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TABLE 1

Average daily protein and vitamin B_{12} content of diets for metabolic studies with 7- to 9-year-old girls

Study and diet[1]	Protein content of diets		Vitamin B_{12} content of diets	
	Total	Animal protein[2]	Analyzed	Calculated
	gm/day	*gm/day*	*μg/day*	*μg/day*
1956				
2	48	33	3.9	3.5
3	73	53	4.5	4.3
4	59	37	4.2	3.7
5	88	53	3.9	4.3
1958				
8	22	10	1.1	1.1
8′	18	6	0.7	0.7

[1] Years of study and diet numbers correspond with those in Southern Cooperative Series Bulletin no. 64 ('59).

[2] Values were calculated from food composition tables (Watt and Merrill, '50) and do not include gelatin which averaged 3, 4, and 19 gm per day for diets 3, 4, and 5, respectively.

in the studies (Harper, '60). The amount of animal protein was calculated from food tables (Watt and Merrill, '50). In diets 2 to 5, about 65% of the total protein (other than gelatin) was from animal sources; whereas in diets 8 and 8′ only about 40% was from animal sources.

Diets 2 and 4 provided daily about 50 and 70 gm of meat, fish, and cheese, respectively; whereas diets 3 and 5 supplied an average of about 140 gm of meat, poultry, fish, eggs, and cheese. All 4 diets also included daily 550 gm of whole milk and about 40 gm of light cream and ice cream. Diets 8 and 8′ provided daily about 15 gm of meat and 80 gm of light cream. Diet 8 included daily 120 gm of whole milk which was omitted from diet 8′.

In table 1 are also presented values for the vitamin B_{12} content of each diet as determined by analysis and as calculated from the vitamin B_{12} content of individual foods of animal origin (Lichtenstein et al., '61). In some instances the determinations for the individual foods were made on raw samples; however, all samples were autoclaved prior to analysis. The values used for calculating the vitamin B_{12} content in each diet are shown below as micrograms of vitamin B_{12} per 100 gm of food:

Beef, round	1.93
Beef, hamburger	1.91
Chicken, boned, canned	0.794
Ham, cured	0.586
Liverwurst	13.9
Pork loin	0.555
Tuna fish, canned, solids	2.80
Cheese, cheddar	0.990
Cream, 20% fat	(0.300)
Egg, whole	3.13
Ice cream, vanilla, 12% fat	(0.398)
Milk, whole	0.362

The imputed value for cream and ice cream was based on the vitamin B_{12} content of the milk.

By analysis, diets 2, 3, 4, and 5 contained an average of 3.9, 4.5, 4.2, and 3.9 μg of vitamin B_{12} per day, respectively (table 1). The low-protein diets, 8 and 8′, contained about one-fourth as much of the vitamin or an average of 1.1 and 0.7 μg per day. The larger amount of vitamin B_{12} in diet 8 could be attributed to the 120 gm of whole milk which was omitted in diet 8′. The calculated values for the vitamin B_{12} content of diets 2, 3, and 4 were 90, 95, and 88%, respectively, of the values determined by analysis. For diet 5, the calculated value was 110% of the analyzed value, whereas diets 8 and 8′ had the same values by calculation and by analysis.

In table 2 data are presented, for selected metabolic periods, on the average daily intake and excretion of vitamin B_{12} for all girls supplied with low-protein diets.

TABLE 2

Vitamin B_{12} intake and urinary excretion by 11 girls receiving controlled diets

Study and diet[1]	Metabolic period	Average vitamin B_{12} intake	Vitamin B_{12} excretion		
			Average	Range	Intake
		μg/day	*μg/day*	*μg/day*	*%*
1958—11 girls					
8	1	1.13	0.10	0.04–0.22	9
8	3	1.11	0.03	0.02–0.04	3
8	5	1.15	0.03	0.02–0.03	3
8′	6	0.63	—	— —	–
8′	8	0.68	0.03	0.02–0.03	4

[1] Year of study and diet numbers correspond with those in Southern Cooperative Series Bulletin no. 64 ('59).

In general, very little vitamin B_{12} was excreted in the urine with these low-protein diets. For the first 6-day period, average daily urinary excretion values ranged from 0.04 to 0.22 μg per day indicating a fivefold variation among the subjects. By the third period at least, the vitamin B_{12} excretions had decreased for all subjects and there was a tendency toward uniform output. In the last metabolic period, after the diet had been supplied for 42 days, the excretions ranged from 0.02 to 0.03 μg per day with less than a twofold variation. In the first metabolic period following the self-chosen diet, the average amount of vitamin B_{12} excreted was 9% of the dietary intake, and in successive periods 3 to 4%.

There was no measurable vitamin B_{12} in the urine for each of 4 girls in period 5 who had received the antibiotic, Cosa-Tetracyn,[2] during this period or in the preceding period. Similar findings had been noted for folic acid when microbiological determinations were made using the test organism *Streptococcus faecalis*.[3] Further investigations indicated that the apparent absence of folic acid was attributable to inhibition of the test organism *S. faecalis* by Cosa-Tetracyn excreted in the urine rather than to lowered excretion of folic acid. A similar explanation may apply to the vitamin B_{12} analyses reported here.

Chow ('51), in a study with normal children, reported no detectable vitamin B_{12} activity in the urine even after daily oral supplements of 25 μg of vitamin B_{12}. Studies with normal adults consuming ordinary diets have shown urinary excretions of vitamin B_{12} which ranged from 0.02 to 0.27 μg per day (Register and Sarett, '51; Mollin and Ross, '52).

The low excretion of vitamin B_{12} in the 1958 study, when the intakes were between 0.68 and 1.15 μg daily, may indicate that practically the full amount of these intakes was needed for cellular maintenance and tissue growth leaving almost none to be excreted unused. This suggests the possibility that an intake of 1 μg of vitamin B_{12} daily may closely approach the minimal requirement for these children.

Diets 2, 3, 4, and 5 were patterned to include largely the usual kinds and amounts of foods needed to meet the Food and Nutrition Board's ('53) recommended allowances for children 7 to 9 years of age, except that protein was above the recommended allowance in diets 3 and 5. Since these diets supplied between 3.9 and 4.5 μg of vitamin B_{12} per day, it could be assumed that the usual nutritionally adequate diet for this age of child would supply about 4 μg of vitamin B_{12} daily.

SUMMARY

The vitamin B_{12} content was determined for each of 6 controlled diets used in a series of metabolic studies with a total of 23 healthy 7- to 9-year-old girls. Urinary vitamin B_{12} excretions were determined for 11 girls receiving two of these diets that were low in protein.

Diets with a daily average of 48 to 88 gm of total protein (about 65% from animal sources) contained from 3.9 to 4.5 μg of vitamin B_{12} per day. Diets of lower protein content with a daily average of 18 and 22 gm of total protein (about 40% from animal sources) contained 0.7 and 1.1 μg of vitamin B_{12} per day, respectively.

The urinary excretion of vitamin B_{12} for the girls receiving low-protein diets was 9% of the dietary intake at the initiation of the controlled diet phase of the study and from 3 to 4% in latter phases.

Diets, made up of ordinary foods patterned to meet the Food and Nutrition Board's recommended allowances for children 7 to 9 years of age, supplied about 4 μg of vitamin B_{12} daily.

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Pantothenic Acid Excretion on Three Levels of Intake[1]

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The pantothenic acid requirement of human subjects has not been established. Estimates of the daily human requirement for pantothenic acid have ranged from 5 mg (Elvehjem, '51) to 10 to 15 mg (Krehl, '53). Work at the University of Iowa (Bean and Hodges, '54; Bean et al., '55a, b; Hodges et al., '58, '59; Lubin et al., '56; Thornton et al., '55) has demonstrated that certain physical and biochemical abnormalities can be produced in man by the omission of the vitamin from the diet or by the addition of a pantothenic acid antagonist. These observations emphasize the desirability of further studies of pantothenic acid metabolism in subjects receiving different amounts of the vitamin.

The purpose of this paper is to present the results of varying the intake of pantothenic acid on its excretion in the urine. These results were obtained in conjunction with a project designed to measure the metabolic response of healthy young women to a controlled diet.

EXPERIMENTAL

Eight women, judged to be in good health on the basis of a medical examination, were subjects. They ranged from 18 to 24 years in age, from 47.4 to 71.7 kg in weight and from 158.7 to 175.9 cm in height.

The study was 40 days in length consisting of 8 consecutive periods of 5 days each and designated A through H. During the first 5 days of the study, period A, the subjects ate their customary self-chosen diets. Quantities of food eaten were recorded in common household measures and the pantothenic acid content of the self-chosen diets was calculated using the tables of Zook et al. ('56) supplemented by those of Sarett et al. ('46).

In periods B through H all subjects were fed the standardized diet developed by the Human Nutrition Research Division of the Agricultural Research Service (Meyer et al., '55). In this diet ordinary foods, providing minimal levels of most nutrients, are combined into palatable meals. The foods are supplemented with purified and synthetic products to bring the nutrient intakes up to moderate levels. Daily intakes of the various nutrients, according to analyses, were: nitrogen, 11.0 gm; fat, 90 gm; calcium, 726 mg; phosphorus, 949 mg; magnesium, 275 mg; ascorbic acid, 62 mg; thiamine, 755 μg; and riboflavin, 833 μg. Other known dietary essentials were calculated to be present in adequate but not excessive amounts.

In table 1 are presented the quantities of pantothenic acid provided during the different experimental periods. The standardized diet provided 2.8 mg of pantothenic acid, 1.8 mg from the food and

TABLE 1

Pantothenic acid intake during various periods

No. of subjects	Period[1]	Intake
		mg
Eight	A	6.7[2] ± 2.1
Eight	B	2.8
Eight	CD	2.8
Four (group 1)	EF	7.8
Four (group 1)	GH	12.8
Four (group 2)	EF	12.8
Four (group 2)	GH	7.8

[1] Each letter represents a 5-day period.
[2] Calculated from self-chosen diet.

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1 mg from calcium pantothenate. The subjects were maintained on the 2.8 mg pantothenic acid intake for 15 days and then divided into two groups, designated as groups 1 and 2. Group 1 received 7.8 mg of pantothenic acid for 10 days, periods EF, followed by 12.8 mg for another 10 days, periods GH. Group 2 received 12.8 mg of pantothenic acid in periods EF and 7.8 mg in periods GH.

Complete collections of urine were made throughout the entire period of study. Urine was covered with toluene and frozen until analyzed.

Pantothenic acid was determined microbiologically on 5-day composites of urine and on individual foods using *Lactobacillus plantarum* as the assay organism (Zook et al., '56). Total pantothenic acid was determined in foods using the double enzyme system of pigeon liver extract and intestinal phosphatase. Free pantothenic acid was determined in urine since preliminary trials indicated there was no bound pantothenic acid present.

RESULTS AND DISCUSSION

The mean daily pantothenic acid intake and urinary excretion of the 8 subjects consuming self-selected and controlled diets are shown in table 2. Period B, the first 5 days that the women received the standardized diet, was considered a transition or adjustment period. Intake and excretion values for this period were not included in calculating the regression equation.

TABLE 2

Means, standard deviations and coefficients of variation for intake and urinary excretion of pantothenic acid in human subjects

	Intake	Urine
	mg/day	*mg/day*
Mean	6.7[1]	3.9
S.D.	2.1	1.5
C.V., %		38
Mean	2.8	3.2
S.D.		0.8
C.V., %		25
Mean	7.8	4.5
S.D.		1.0
C.V., %		22
Mean	12.8	5.6
S.D.		0.6
C.V., %		11

[1] Calculated from self-chosen diet.

The mean calculated pantothenic acid content of the self-selected diets in period A was 6.7 mg, with individual diets providing from 3.4 to 10.3 mg. In general, the pantothenic acid intake paralleled the intake of other nutrients. The diets of 5 subjects which met or exceeded the National Research Council ('58) recommendations for other nutrients provided approximately 7 mg of pantothenic acid daily, whereas diets of two subjects in which other nutrients were present in less than recommended quantities provided between 3 and 4 mg of pantothenic acid.

The mean urinary excretion of pantothenic acid with the self-selected diets was 3.9 mg daily, individual values ranging from 2.9 to 7.5 mg. Twenty-four-hour excretion values reported by other investigators for individuals consuming self-selected diets are of similar magnitude: 1.2 to 4.5 mg (Schmidt, '51); 1.6 to 3.5 mg (Scuro, '53); and 2.5 to 9.6 mg (Fitzpatrick and Tompsett, '50).

Pantothenic acid excretion responded to changes in intake. The lowest amount of pantothenic acid fed during the controlled periods was 2.8 mg, an amount which was less than that received by any subject eating her self-chosen diet. The urinary excretion of each subject decreased during the three 5-day periods on the standardized diet. Urinary excretion, however, exceeded the 2.8 mg pantothenic acid intake, indicating that either previous body stores were being lost or that synthesis was occurring. Raising the pantothenic acid intake to either 7.8 or 12.8 mg daily in subsequent periods resulted in an increase in excretion, and with these higher intakes the urinary excretion did not exceed the intake. With an intake of 7.8 mg of pantothenic acid the subjects excreted from 3.4 to 6.3 mg daily, mean, 4.5 mg; with an intake of 12.8 mg, from 5.2 to 7.1 mg with a mean of 5.6 mg. These excretions represented 58 and 44% of the 7.8 and 12.8 mg intakes, respectively. That the subjects excreted 100% or more of the 2.8 mg pantothenic acid intake and only about 50% of the higher amounts suggests that 3.2 mg, the excretion associated with the lower intake, may

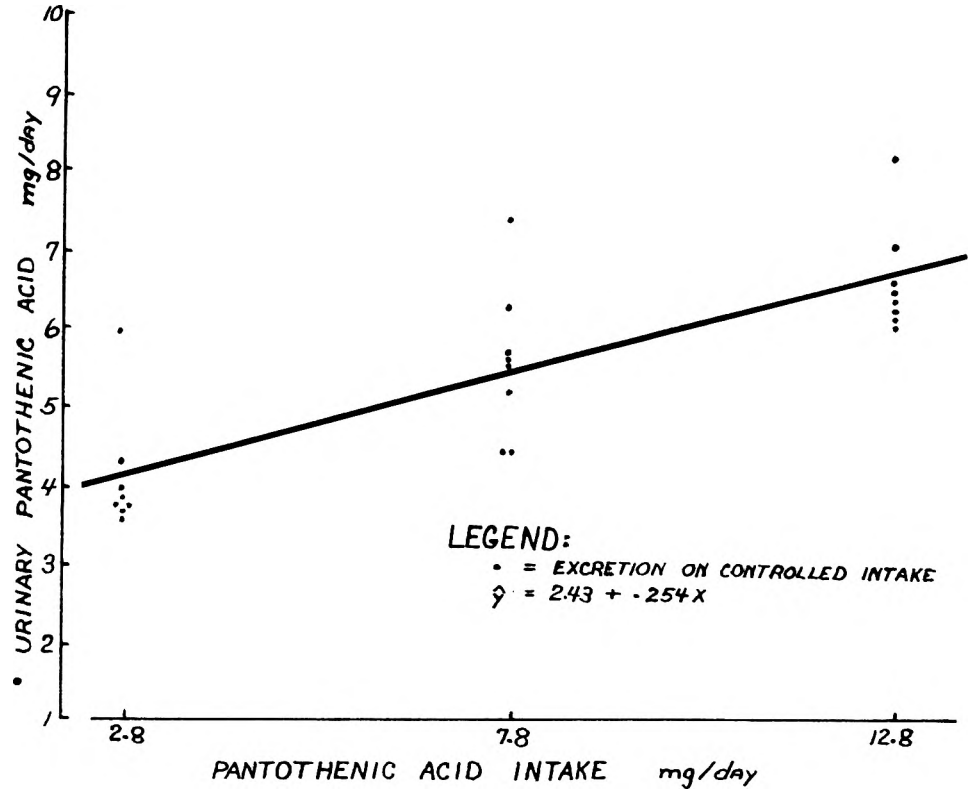

Fig. 1 Relation of intake to urinary excretion of pantothenic acid with controlled diets.

represent the minimal daily requirement for tissue maintenance. Moreover, excretion was always less than intake when the diet provided 7.8 mg of pantothenic acid, an amount similar to that provided by self-selected diets containing recommended quantities of other nutrients, suggesting that 7 to 8 mg of pantothenic acid was a desirable intake for these subjects.

The pantothenic acid excretion of each subject at each level of intake of the controlled diet is shown in figure 1. The regression of excretion on intake is highly significant, $r = 0.805$ ($P < 0.01$).

The urinary excretion of pantothenic acid was influenced somewhat by the sequence in which the subjects received the different levels of pantothenic acid. Although both groups excreted the largest amount of the vitamin on the highest intake, the difference in excretion between the 7.8 and 12.8 mg intakes was 1.6 mg for the 4 subjects who received 7.8 mg followed by 12.8 mg, but only 0.6 mg for subjects receiving 12.8 mg followed by 7.8 mg. Corresponding increments in excretion between the 2.8 and 7.8 mg intakes were 1.0 mg for the group that received the lower amount first and 1.8 mg for the group that received the higher amount first. These results indicate that on the higher intakes, appreciable amounts of the vitamin were being retained by the body.

The amount of pantothenic acid stored in the body may explain in part the difficulty in producing pantothenic acid deficiency in man. Hodges et al. ('58) maintained human subjects with highly purified pantothenic acid deficient diets for 12 weeks before observing symptoms of fatigue, malaise and mental change. The mean urinary pantothenic acid fell to less than 1 mg daily. Luckey et al. ('55) in a study of germ-free rats reported that 90% of the pantothenic acid fed or originally present in the animals was observed in the excreta and carcasses. The

authors interpret this finding as indicating that pantothenic acid is not metabolized or broken down in the body.

The level of pantothenic acid in the diet did not affect significantly the urinary and fecal excretion of nitrogen, calcium, phosphorus or magnesium, the fecal excretion of fat, or the urinary excretion of thiamine or riboflavin, according to statistical analysis of the results.

SUMMARY

Urinary excretion of pantothenic acid was measured in response to self-chosen diets and to a controlled standardized diet providing 2.8, 7.8 and 12.8 mg of pantothenic acid daily. Each subject was studied for 10 days at each level of pantothenic acid intake following an adjustment period using the standardized diet. Excretion of pantothenic acid responded to changes in intake. The mean excretion on intakes of 2.8, 7.8 and 12.8 mg of pantothenic acid daily was 3.2, 4.5 and 5.6 mg, respectively. Comparison of the excretion of the subjects fed the higher levels of pantothenic acid in different order indicates that urinary excretion may reflect previous dietary intake of the vitamin.

The calculated pantothenic acid content of quantitatively estimated self-chosen diets that were adequate with respect to other nutrients was approximately 7 mg daily. The mean pantothenic acid excretion by subjects consuming these self-chosen diets was 3.9 mg daily.

Altering the pantothenic acid intake from 2.8 to 12.8 mg daily did not affect the excretion of other nutrients.

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Sulfur Amino Acid Requirements for Growth of Mice Fed Two Levels of Nitrogen

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The studies of Totter and Berg ('39) and Bauer and Berg ('43) have established the amino acids essential for growth of the mouse. In these and later studies (Celander and Berg, '53), the ability of the optical isomers of amino acids to support growth in the mouse was studied. A paucity of information exists, however, concerning the quantitative amino acid requirements for the growing mouse.

The amino acid diets used by Bauer and Berg ('43) did not support a rate of gain equivalent to that of mice fed a casein diet. Although the report of Maddy and Elvehjem ('49) presented an amino acid diet which supported growth equivalent to that obtained with a casein diet, quantitative needs for the individual amino acids were not studied.

The present investigations were undertaken in an effort to develop a casein-amino acid diet that would support maximal growth in the mouse, and yet would supply sufficiently low levels of the essential amino acids so that the diet could be used for the determination of the quantitative amino acid requirements for growth. This report presents data on the use of this diet in the determination of the sulfur amino acid requirement of the weanling mouse.

EXPERIMENTAL

Male, albino mice of the Swiss Webster strain were used in all studies. Weanling mice, weighing from 8 to 12 gm were housed (4 mice per cage) in wire cages with raised wire floors, in a temperature-controlled room. The animals were fed the experimental diets and water ad libitum for a two-week experimental period. The mice were weighed and food consumption was determined weekly.

The composition of the basal diet used was as follows (in gm/100 gm of diet): salt mixture (USP XIV), 4.0; vitamin mixture,[1] 0.4; choline chloride, 0.3; fiber, 4.0; corn oil, 5.0; starch, to 100. The nitrogen was supplied by the following mixture (gm per 100 gm of mix): casein (14.89% N), 22.18; L-arginine·HCl, 6.64; L-histidine·HCl·H_2O, 2.09; L-lysine·HCl (95% purity), 4.95; DL-tryptophan, 1.10; DL-phenylalanine, 7.48; DL-threonine, 3.08; L-leucine, 2.20; DL-isoleucine, 1.76; DL-valine, 4.51; L-glutamic acid, 44.00. This mixture was added to the basal diet at the rate of 18.18 and 10.91 gm per 100 gm of diet to obtain the 2.5 and 1.5% nitrogen levels, respectively. The final adjustment of the nitrogen levels was made with DL-methionine, L-cystine and glycine in such a manner as to maintain the diets isonitrogenous at the various methionine levels employed. The casein present in these diets supplied 24% of the total nitrogen. The basal diets contained (by calculation) 0.133% of methionine and 0.028% of cystine at the 2.5% nitrogen level, and 0.080% of methionine and 0.017% of cystine at the 1.5% nitrogen level (Block and Weiss, '55). The amino acid composition of the basal diet was patterned after the diets of Maddy and Elvejhem ('49).

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[1] The vitamin mixture supplied, when fed at the rate of 4.0 gm/kg of diet, the following amounts: thiamine·HCl, 22.0 mg; riboflavin, 22.0 mg; Ca pantothenate, 66.0 mg; pyridoxine, 22.0 mg; biotin, 0.44 mg; folic acid, 2.0 mg; *p*-aminobenzoic acid, 110 mg; 2-methyl-1,4-naphthoquinone, 50 μg; vitamin B_{12}, 30 μg; inositol, 110 mg; ascorbic acid, 1.0 gm; niacin, 100 mg; vitamin A, 10,000 IU; vitamin D_2, 2,000 IU; α-tocopheryl acetate, 50 mg.

RESULTS

The growth rate of mice fed the amino acid diet supplemented with 0.77% of DL-methionine and supplying 2.5% of nitrogen was compared to that of animals receiving a diet supplying the same level of nitrogen from casein and 0.3% of L-cystine. Weanling mice fed the amino acid and casein diets gained 7.3 ± 1.34 and 8.2 ± 1.14 gm (mean of 10 mice ± standard deviation) in two weeks, respectively. Since the difference observed between the two groups was not statistically significant, the amino acid diet was considered to be equivalent to the isonitrogenous casein diet.

The influence of feeding various levels of supplemental methionine, in diets supplying either 2.5 or 1.5% nitrogen, on weight gain of weanling mice is demonstrated by the data presented in table 1 and figures 1A and 1B. At the 2.5% nitrogen level, a linear response to supplemental methionine was observed up to the 0.30% level; further supplementation failed to evoke a statistically significant increase in weight gain. The requirement under these conditions was estimated by plotting the two-week weight gains against level of supplemental methionine, calculated for the 6 lower levels of methionine fed. The point at which the least squares line for this plot intersected a horizontal line, representing the mean weight gain for the three highest levels of methionine fed, was taken as the requirement. This plot is presented in figure 1A. The point at which the regression line ($Y = 29.77 X - 0.653$, where Y = weight gain in grams and X = percentage of supplemental methionine) intersects the horizontal line corresponds to 0.32% of supplemental methionine. This level represents the lowest level of supplemental methionine that resulted in maximal growth with the diet used. The estimated sulfur amino acid requirement for maximal growth at a nitrogen intake level of 2.5% of the diet, taking into account the methionine (0.13%) and cystine (0.08%) supplied by casein, was 0.53% of the diet.

TABLE 1

Influence of supplemental DL-*methionine on growth of weanling mice receiving either a 2.5 or a 1.5% nitrogen diet*[1]

Added DL-methionine	2-Week gain	
	2.5% N	1.5% N
% of diet	*gm*	*gm*
0.00	−0.8 ± 1.34[2](10)[3]	−1.4 ± 1.18(12)
0.05	0.9 ± 1.79 (12)	0.4 ± 1.30(12)
0.10	2.2 ± 1.83 (24)	3.0 ± 1.49(24)
0.15	4.3 ± 1.52 (11)	4.2 ± 0.87(12)
0.20	5.1 ± 1.23 (24)	4.0 ± 1.40(24)
0.30	8.2 ± 1.86 (12)	5.0 ± 2.04(12)
0.40	8.7 ± 2.56 (12)	5.9 ± 1.79(10)
0.50	9.4 ± 1.08 (12)	4.2 ± 2.44(12)

[1] Basal diet calculated to contain 0.133 and 0.080% methionine and 0.028 and 0.017% cystine at the 2.5 and 1.5% N levels, respectively.
[2] Mean ± standard deviation.
[3] Values in parentheses represent the number of animals.

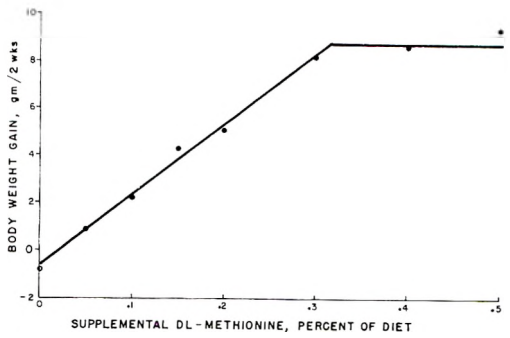

Fig. 1A Influence of DL-methionine level in a diet supplying 2.5% of nitrogen on weight gain of the weanling mouse.

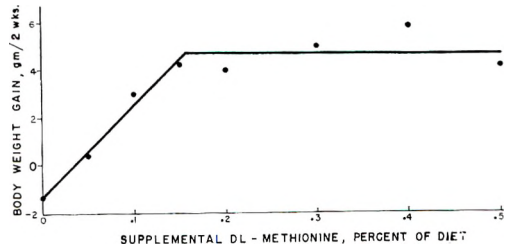

Fig. 1B Influence of DL-methionine level in a diet supplying 1.5% of nitrogen on weight gain of the weanling mouse.

The influence of methionine supplementation on the weight gain of mice fed the 1.5% nitrogen-containing diet can be seen from the data presented in table 1. There was a linear increase in weight gain as a result of methionine supplementation up to a level of 0.15% of the diet. The requirement for maximal growth at this nitrogen level was estimated as previously

described for the 2.5% nitrogen diet. The point at which the least squares line, calculated for the 4 lowest levels of methionine supplementation, intersected the horizontal line, drawn through the mean weight gain of the mice receiving the 5 highest levels of methionine, was calculated from the regression equation ($Y = 38.725\ X - 1.354$, where Y = two-week weight gain in gm and X = percentage of supplemental methionine). The plot used for estimating the requirement is presented in figure 1B. The estimated requirement was 0.16% of supplemental methionine; when the methionine and cystine supplied from casein are considered, 0.028 and 0.017%, respectively, the total sulfur amino acid requirement was estimated to be 0.20% of the diet.

The data presented in table 2 demonstrate the lack of effect of supplemental methionine on weight gain when the diet was supplemented with 0.3% of L-cystine. There were no significant differences at either nitrogen level due to methionine supplementation beyond the 0.10% level.

DISCUSSION

The data presented demonstrate a greater requirement for sulfur amino acids in animals fed a 2.5% nitrogen-containing diet as compared with one containing 1.5% of nitrogen. This increased requirement is in accord with observations in other species (Almquist, '59). The requirement when expressed as a percentage of the protein (N × 6.25) level is higher at the higher nitrogen level, an observation which is in contrast to observations in the chick (Almquist, '59) which demonstrate a lower requirement, expressed as a percentage of the protein, at higher levels of protein intake.

The lack of a response to supplemental methionine in mice fed diets containing 0.3% of cystine indicates that this amino acid can meet over one-half of the total sulfur amino acid requirement at a dietary nitrogen level of 2.5%. That cystine cannot meet the total requirement for sulfur amino acids in the mouse is demonstrated by the studies of Sauberlich ('59), which show that a methionine-free diet supplemented with 0.3% of L-cystine results in weight loss and death which can be overcome by the addition of methionine.

TABLE 2

Influence of supplemental DL-*methionine on growth of weanling mice receiving either a 2.5 or 1.5% nitrogen diet containing 0.3% added* L-*cystine*[1]

Added DL-methionine	2-Week gain	
	2.5% N	1.5% N
% of diet	*gm*	*gm*
0.10	9.0 ± 3.15[2]	5.5 ± 2.46
0.20	9.6 ± 3.12	6.2 ± 1.36
0.30	9.9 ± 2.54	6.7 ± 1.12
0.40	10.8 ± 1.92	5.6 ± 1.52
0.50	10.8 ± 1.59	5.9 ± 2.12

[1] Basal diet calculated to contain 0.133 and 0.080% methionine, and 0.028 and 0.017% cystine at the 2.5 and 1.5% nitrogen levels, respectively.

[2] Mean for 12 mice ± standard deviation.

SUMMARY

A casein-amino acid diet was developed that will sustain growth in the mouse equivalent to that obtained with an isonitrogenous, cystine-supplemented casein diet.

The casein-amino acid diet was used to determine the total sulfur amino acid requirement for maximal growth in the weanling mouse at two nitrogen levels, 2.5 and 1.5% of the diet. The estimated requirement for total sulfur amino acids at the 2.5 and 1.5% levels of nitrogen was 0.53 and 0.20% of the diet, respectively.

Data were presented which indicate that at the 2.5% nitrogen level cystine can meet over one-half of the total sulfur amino acid requirement.

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The Interrelationships Between Dietary Molybdenum, Copper, Sulfate, Femur Alkaline Phosphatase Activity and Growth of the Rat[1]

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Molybdenosis, molybdenum toxicity in animals, has recently been reviewed (Dick, '56; Underwood, '56; Miller and Engel, '60). Animals grazing forages grown on high-molybdenum-content soils developed a condition which included diarrhea, emaciation, achromotrichia, and weakness. Ferguson et al. ('38) first observed that molybdenosis was lessened by dietary copper. Inorganic sulfate was first observed to alleviate molybdenosis by Dick ('53). Mandibular and maxillary exostosis was observed in the rat fed molybdenum-toxic diets, a condition mitigated by dietary inorganic sulfate (Van Reen, '59).

Using the rat and other laboratory animals, bone abnormalities, attributable to molybdenosis, have been observed (Comar et al., '49; Arrington and Davis, '53; Miller et al., '56).[3] Increased bone alkaline phosphatase activity has been observed concomitantly with an increase in the ossification process (Martland and Robison, '27; Kay, '32; Wilkins and Regen, '35). Molybdenosis decreases the activity of alkaline phosphatase in the kidney (Van Reen and Williams, '56; Mills et al., '58) and intestine (Williams and Van Reen, '56) and increases the activity of the enzyme in liver (Van Reen, '54; Mills et al., '58). A decrease in femur alkaline phosphatase with molybdenosis has been observed.[4]

The objective of this study was to observe the effects of dietary molybdenum, copper, and inorganic sulfate upon the femur alkaline phosphatase activity of the rat. Attention was also given to the effects of dietary restriction on enzyme activity.

METHODS

Male weanling albino rats, of the Sprague-Dawley strain,[5] were housed individually in stainless steel cages, with wire floors. It has been observed that the Sprague-Dawley strain of rat was more susceptible to molybdenosis than the Holtzman strain using weight gain as an indication of the degree of molybdenosis.[6] The rats were housed in an animal room without facilities for constant temperature and humidity. Eight rats were randomly assigned to each treatment lot in all experiments except experiment 2. In experiment 2, 64 rats were randomly assigned to two equal lots and each lot was further divided into 8 sub-lots of 4 rats. At the initiation of the experiment, one sub-lot from each lot was selected for each sacrificing (after zero, 1, 2, 3, 4, 5, 6 and 8 weeks of feeding).

A purified diet as described by Miller et al. ('56) was fed and contained in per cent: sucrose, 81.5; vitamin-free casein,[7] 10; fat,[8] 5; mineral mix, 2.5; and vitamin mix, 1. Food and tap water were

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[2] Submitted in part as an M. S. Thesis by Herman L. Johnson to the Department of Biochemistry and Nutrition at Virginia Polytechnic Institute, Blacksburg, Virginia.
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[8] Wesson Oil, Wesson Oil Company, New Orleans, Louisiana.

provided ad libitum. When indicated, molybdenum, copper, or sulfate was added to the diet as an aqueous solution of $Na_2MoO_4 \cdot 2H_2O$, $CuCl_2$, or equimolar K_2SO_4: Na_2SO_4, respectively. The basal diet was a low sulfate (less than 3.0 ppm of sulfate added) diet containing 3.2 ppm of copper and less than 0.1 ppm of molybdenum. At the end of the experimental period, the rats were decapitated and the femurs removed.

A crude enzyme extract was prepared from the femurs by the method of Martland and Robison ('29) using 10 ml of ion-free water per gm of fresh bone. A modification of the method Bessy et al. ('46) was used to determine the femur alkaline phosphatase activity. Disodium phenyl phosphate, 0.001 M, containing 0.009 M Mg^{++} and buffered at pH 10.0 ± 0.05, was incubated at 37° for 20 minutes with the enzyme. In the first experiment, 0.05 M glycine was used as the buffering agent but thereafter 0.02 M sodium barbital with 0.00625 M glycine was used. Bodansky ('46) reported that glycine and other amino acids noncompetitively inhibited bone alkaline phosphatase. All assays were made in duplicate and the phenol liberated was determined colorimetrically by the method of Folin and Ciocalteau ('27). The enzyme activity was expressed as micromoles of phenol liberated per hour per gram of fresh demarrowed bone.

All tables and figures reported in this paper are based upon the enzyme activity of the right femurs. All statistical analyses were made using the *t* test, single degree of freedom contrasts, analysis of variance (Snedecor, '56), and multiple range tests as modified by Kramer ('56).

RESULTS AND DISCUSSION

The first experiment was designed to find the optimal level of molybdenum to be used in the diets in order to observe a significant depression in alkaline phosphatase activity. Three levels of molybdenum were fed. The effect of molybdenosis upon weight gain (table 1) has been well documented in this and other laboratories. A significant ($P < 0.01$) decrease in femur alkaline phosphatase activity was observed after feeding molybdenum for 6 weeks (table 1), a larger decrease was observed with the higher level of molybdenum fed. Using glycine, the noncompetitive inhibitor, as the buffering agent in the enzyme assays, there was a significant difference in enzyme activity of right versus left femurs.[9] In later experiments using sodium barbital as the buffering agent, this right-left difference in enzyme activity was not observed.

TABLE 1

Effect of level of dietary molybdenum upon femur alkaline phosphatase activity and weight gain (8 rats/treatment)

Mo added	Av. weight gained in 6 weeks	Femur alkaline phosphatase activity[1]
ppm	*gm*	
0	182.8 ± 18.7[2]	762.2 ± 56.6[2]
300	60.6 ± 9.9	532.9 ± 81.2
600	18.2 ± 8.3	434.5 ± 113.7

[1] Expressed as micromoles of phenol liberated per hour per gram of fresh demarrowed bone.
[2] Standard deviation about the mean.

The second experiment was designed to ascertain the length of the molybdenum feeding period necessary before a significant depression of the enzyme activity was detectable and to determine the length of the feeding period necessary for the maximal depression in activity. One lot of rats was fed the basal diet and the other the basal plus 600 ppm of molybdenum. The results are shown in figure 1 (experiment 2-A). There appeared to be an increase in femur alkaline phosphatase activity (significant at $P < 0.01$) after one

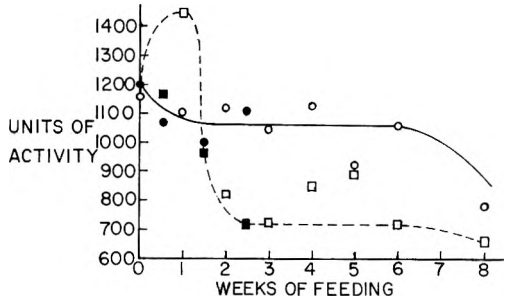

Fig. 1 Effect of molybdenum feeding period upon femur alkaline phosphatase activity. Open circles, basal diet fed (−Mo) in exp. 2-A. Open squares, basal diet + 600 ppm Mo fed in exp. 2-A. Closed circles, basal diet fed in exp. 2-B. Closed squares, molybdenum fed in exp. 2-B.

[9] See footnote 6.

week of molybdenum feeding. The femur alkaline phosphatase activity of rats fed the basal diet (— Mo) remained essentially constant for 6 weeks and then decreased markedly. A later experiment (experiment 2-B, fig. 1), sacrificing 8 animals per treatment at zero, 3, 10, and 17 days of feeding, confirmed the molybdenum-induced decrease of enzyme activity between 10 and 17 days. The increase of activity observed after one week of molybdenum feeding in experiment 2-A was not noted at either three or 10 days of molybdenum feeding. The differences in enzyme activity between the basal diet fed and the basal diet plus 600 ppm of molybdenum fed rats were statistically significant ($P < 0.01$) at all times after two weeks of feeding except at the 5th week and the authors believe that this nonsignificant difference may be due to the small number of observations. Results of these experiments justified the subsequent use of a 21-day feeding period.

The objective of experiment 3 was to determine the effect of added dietary copper upon the activity of femur alkaline phosphatase. Four levels of copper were fed with the basal diet and the basal diet plus 600 ppm of molybdenum. The results are summarized in table 2. Added dietary copper, up to 50 ppm, did not affect either weight gain or enzyme activity whether molybdenum was fed or not.

TABLE 2

Effect of dietary molybdenum and copper upon femur alkaline phosphatase and weight gain (8 rats/treatment)

Added dietary Cu	Enzyme activity[1] ± SD	Av. weight gained in 3 weeks ± SD
ppm		*gm*
	No molybdenum	
0	974.1 ± 135.4	76.4 ± 8.0
12.5	1002.6 ± 186.9	74.1 ± 11.8
25	1002.9 ± 91.6	77.8 ± 8.2
50	1036.1 ± 93.3	83.6 ± 11.1
	600 ppm Molybdenum	
0	850.9 ± 141.0	8.6 ± 4.0
12.5	767.8 ± 110.4	5.5 ± 3.7
25	828.9 ± 117.1	8.8 ± 4.3
50	790.6 ± 123.0	6.6 ± 5.7

[1] Expressed as micromoles of phenol liberated per hour per gram of fresh demarrowed bone.

TABLE 3

Effect of dietary molybdenum and sulfate upon femur alkaline phosphatase activity and weight gain (8 rats/treatment)

Dietary SO_4	Enzyme activity[1] ± SD	Av. weight gained in 3 weeks ± SD
ppm		*gm*
	No molybdenum	
0	1085.4 ± 107.6	58.2 ± 7.4
250	1048.8 ± 76.4	63.6 ± 5.3
500	980.0 ± 63.8	69.1 ± 4.9
750	1028.1 ± 63.4	70.5 ± 12.0
1500	1024.3 ± 102.5	53.8 ± 12.4
	600 ppm molybdenum	
0	731.1 ± 136.6	1.4 ± 4.9
250	920.0 ± 129.8	21.4 ± 8.1
500	1024.7 ± 138.6	29.1 ± 5.7
750	1040.7 ± 142.4	29.4 ± 7.8
1500	1019.3 ± 146.4	23.1 ± 6.2

[1] Expressed as micromoles of phenol liberated per hour per gram of fresh demarrowed bone.

Experiment 4 had as its objective the determination of the effect of dietary inorganic sulfate upon the molybdenum-induced depression of femur alkaline phosphatase activity. Five levels of sulfate were fed with the basal diet (— Mo) and the basal diet plus 600 ppm of molybdenum. The results are summarized in table 3. Added dietary sulfate had no significant effect upon either weight gain or femur alkaline phosphatase activity of the rats fed the basal diet (— Mo). Dietary sulfate, 500 ppm, completely alleviated the molybdenum-induced depression in enzyme activity and partially alleviated the growth depression. Higher dietary levels of sulfate did not exert any additional effect upon either enzyme activity or growth of the molybdenum-fed rat.

To determine whether the depression in enzyme activity was due to the ingestion of molybdenum per se or was the result of the generally poor growth and condition of the molybdenum-fed rat, a paired-feeding experimental design was used. Precautions were taken to minimize feed wastage. Using three treatment lots of rats in the conventional paired-feeding experiment (experiment 5-A, table 4), the data indicated that nearly half of the reduction in enzyme activity was due to feed restriction and the resultant reduced rate of growth. Since the weight gain of the

TABLE 4

Effect of restricted food consumption upon femur alkaline phosphatase activity

	Basal fed ad libitum	Basal + 600 ppm Mo ad libitum	Basal, pair-fed	Basal, restricted-fed
Exp. 5-A				
Average food consumed in 3 weeks, gm	216.70 ± 34.03	90.60 ± 13.29	94.87 ± 14.20	
Average weight gained, gm	70.5 ± 12.8	1.8 ± 1.7	21.9 ± 6.5	
Enzyme activity[1]	1272.1 ± 57.2	774.1 ± 120.2	1079.6 ± 115.6	
Exp. 5-B				
Average food consumed in 3 weeks, gm	178.09 ± 21.24	85.42 ± 12.22	85.34 ± 11.22	52.26 ± 2.09
Average weight gained, gm	58.2 ± 7.4	1.4 ± 4.9	16.2 ± 6.4	1.4 ± 4.8
Enzyme activity	1085.4 ± 107.6	731.1 ± 136.6	1018.2 ± 122.0	810.9 ± 130.0
Exp. 5-C				
Average food consumed in 3 weeks, gm	165.43 ± 24.19	65.93 ± 10.93	69.62 ± 11.20	42.00 ± 0.00
Average weight gained, gm	53.1 ± 8.4	−3.8 ± 2.9	14.0 ± 6.4	−4.8 ± 3.3
Enzyme activity	998.3 ± 114.6	548.2 ± 111.3	769.8 ± 117.5	592.8 ± 122.9

[1] Expressed as micromoles of phenol liberated per hour per gram of fresh demarrowed bone.

pair-fed rats exceeded that of the molybdenum-fed rats, the experiment was repeated and a 4th group of rats was added for which the food consumption was adjusted so as to achieve the same rate of growth as that of the molybdenum-fed rats (experiment 5-B, table 4). From these data, it is clear that the depression in enzyme activity can be explained as resulting from complete cessation of growth and not from molybdenum feeding per se; also that the efficiency of feed utilization was definitely depressed by dietary molybdenum. In the molybdenum-fed rat, 85.4 gm of feed were necessary for weight maintenance, whereas only 52.3 gm (62% as much) were necessary for weight maintenance in the restricted-fed rats. The experiment was repeated (experiment 5-C) with similar results—the restricted-fed rats requiring 63% as much feed as the molybdenum-fed rats. Whether this constitutes interference with absorption or digestion of feed constituents or other derangements in metabolism has not been determined. Gray and Daniel ('54) reported similar effects of dietary molybdenum upon feed utilization.

TABLE 5

Statistical correlations of the means of the enzyme activity and weight gain of the rat (8 values/mean)

No. of means	a[1]	β[2]	r[3]
11[4]	729.2	7.22	0.87
8[5]	690.7	16.60	0.90
19[6]	711.3	13.83	0.86

[1] Enzyme activity with no weight gain.
[2] Slope of enzyme activity versus weight gain.
[3] Correlation coefficient.
[4] All means from exp. 5.
[5] All means from exp. 5 with $x < 30$.
[6] Means from exp. 3, 4, and 5 with $x < 30$.

Statistically analyzing the means of the weight gain, x, and enzyme activity, y, for a linear regression, the data in table 5 were obtained. From these data, it is apparent that there is a high degree of correlation between the enzyme activity and the three-week weight gain according to the equation, $y = \alpha + \beta x$. This high correlation is observed only when the means of the treatment groups are treated. The large variation between individual observations made it impossible to show any significant correlation between weight gain and enzyme activity of individual rats within or between treatment groups. The high β's for $x < 30$ gm of gain indicate that with a highly reduced rate of growth, a small amount of gain has a large influence upon the level of enzyme activity. Therefore, the restoration of enzyme activity in the molybdenum-fed rats receiving supplementary sulfate may be attributed to the improvement in growth and likewise, supplementary copper did not restore the enzyme activity since it

did not improve growth. Siegel and Monty ('61) reached a similar conclusion regarding the effect of molybdenum upon sulfide oxidase. In their experiments, the weight gain of the pair-fed animals closely paralleled those of the animals receiving molybdenum, and the reduction in sulfide oxidase activity was attributed to the reduced feed consumption of the molybdenum-fed rats.

From these studies, it can be concluded that bone alkaline phosphatase activity is rather closely related to growth. When growth was eliminated as a variable, there was no effect of dietary molybdenum on this enzyme. The principal consequence of molybdenum-feeding appeared to be a reduction in efficiency of feed utilization and the mechanism involved requires further study.

SUMMARY

1. Feeding 600 ppm of molybdenum resulted in a significant ($P < 0.01$) depression of femur alkaline phosphatase activity and growth of the rat.

2. This depression in activity occurred between 10 to 17 days of molybdenum feeding and was evident up to 8 weeks of molybdenum feeding.

3. Added dietary inorganic sulfate alleviated the molybdenum-induced depression in enzyme activity and partially mitigated the growth depression, whereas added copper had no effect.

4. Through controlled feed intake studies, it was established that the decrease in enzyme activity, induced by molybdenum-feeding, was the result of the growth depression and not the ingestion of molybdenum per se.

5. Dietary molybdenum, 600 ppm, completely suppressed growth. Equivalent growth depression was achieved by restricting the basal ration fed to about 62% of that consumed by the molybdenum-fed animals. Thus, a reduction in the efficiency of feed utilization is one of the important consequences of molybdenosis in the rat. Whether this loss in efficiency is effected through faulty digestion or absorption of nutrients or through some other derangement in metabolism remains to be determined.

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