Effect of Amino Acid Imbalance on Nitrogen Retention

I. EFFECT OF A RELATIVE DEFICIENCY OF TRYPTOPHAN IN DOGS[1,2]

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One of the principal concepts emerging from nutrition research is that the nutritive value of a protein is determined by the relative proportions of its amino acids. In the amino acid supplementation studies of Elvehjem ('56), Harper ('57–'58, '59) and Salmon ('58) have emphasized the concepts of amino acid imbalance, amino acid antagonism, and amino acid toxicity. In most of the amino acid imbalance studies, the overall effect of imbalance is determined by measuring the total change in body weight when amino acid mixtures of food proteins deficient in one or more essential amino acids are added to low-protein diets. It is becoming increasingly evident, however, that significant exchanges in nitrogen compounds among individual tissues may occur without any accompanying change in the total body weight of the animals.

A second approach is the effect of amino acid imbalance on nitrogen balance. The two studies of this type, all of them with rats, (Deshpande et al., '58; Kumta, et al., '58) indicated that an amino acid imbalance decreases the percentage retention of the absorbed nitrogen. Recently, Harper ('59) showed that addition of gelatin, a protein deficient in tryptophan, to casein supplemented with methionine caused an amino acid imbalance which can be corrected by tryptophan supplementation. The experiments to be reported apply to the nitrogen balance methods, to the measurement of the effects of imbalance of young growing dogs.

MATERIALS AND METHODS

The amino acid imbalance was produced by adding an imbalanced protein, gelatin, to a small amount of another protein, casein, having only minor amino acid deficiencies. For the basal diet the deficiencies were corrected by adding the deficient amino acids, tryptophan and methionine. For the experiments, three young, growing female Doberman dogs with initial weights of 4.21, 4.79 and 4.19 kg were used. The animals were freed of parasites and placed in individual metabolism cages. After a 7-day adjustment period, they were fed the experimental diets. The basal diet contained: (in per cent) casein (vitamin-free), 8; gelatin, 15; DL-methionine, 0.3; DL-tryptophan, 0.2; hydrogenated vegetable fat, 10; mineral mixture (Hegsted et al., '41), 2; cod liver oil, 1; powdered cellulose,[3] 2.7; dextrose, 23; sugar, 15, and dextrin, 22.8. The diet was supplemented with 4 ml of a complete vitamin mixture (Manna et al., '53). Imbalances were induced by omitting either one of the amino acids at a time or both from the basal diet.

In the first experiment the animals were fed the basal diet, before and after the basal diet without tryptophan, at a level of 6 gm of protein and 150 Cal. per kg per day. In the second experiment, the basal diet was fed before and after feeding the basal without methionine, at a protein intake of 5 gm per kg per day and the caloric intake was 120. In the third experiment, the animals were fed approximately 4.9 gm of protein and 119 Cal. per kg per day in the following diet sequence: the basal, basal without tryptophan, basal without tryptophan and methionine, basal without methionine, and basal. Each was fed for

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

three consecutive 4-day balance periods with two days for adaptation after a diet change. The diet, moistened with warm water before feeding, was given twice a day, at 8 AM and 4 PM. Water was available at all times. The animals were weighed daily. Feces and urine were collected twice a day and stored at 4°C until analysis was performed. Urine was collected with 1 cm^3 of concentrated acetic acid. Diets, feces, and urine were analyzed for nitrogen by the Kjeldahl method.

RESULTS

Experiment 1. Table 1 shows the results of the first experiment in which tryptophan was omitted from the basal diet. Average nitrogen retention with the basal diet was 27.4% of the nitrogen intake. When tryptophan was omitted, nitrogen retention decreased to an average of 18.8%, with the second 4-day period showing the highest nitrogen retention and the third giving the lowest. A significant increase to 35.1% in nitrogen retention of the intake occurred when the dogs were fed the basal diet again. All nitrogen balances with this treatment were higher than those observed with the same diet before the imbalance was introduced by omitting tryptophan from the diet.

Experiment 2. Table 2 presents the results of nitrogen balance experiments in which methionine was omitted from the basal diet. Nitrogen retention with the basal diet averaged 38.9% of the nitrogen intake. When methionine was omitted from the basal diet, nitrogen balance decreased to an average of 17.6%. The first period gave the lowest nitrogen retention, and the second the highest. Correction of the imbalance by adding methionine resulted in an increase in nitrogen retention averaging 35.9% of the nitrogen intake. Little variation in nitrogen balance was observed in all three periods.

Experiment 3. As shown in table 3, the basal diet resulted in an average nitrogen retention of 32.8% of the nitrogen intake. The omission of tryptophan resulted in a decrease in nitrogen balance to 18.5% a response equal to that observed in experiment 1. The omission of both amino acids together increased nitrogen balance to an average retention of 24.7% with relatively little variation among the three 4-day periods. The feeding of the basal diet without methionine decreased nitrogen retention progressively from the first to the third period with a resulting average value of 15.8%. The terminal feeding of the basal diet resulted in a significant increase

TABLE 1

Effect on nitrogen balance[1] on the omission of tryptophan from the basal diet

Period no.[2]	Nitrogen				Gain in wt
	Intake	Fecal	Urine	Retention	
	mg/kg/day			*% of intake*	*gm*
	Basal diet				
1	1,075	35	805	21.9	64
2	1,049	22	711	30.1	154
3	1,011	49	656	30.3	139
Avg	1,045	35	724	27.4	119
	Basal diet minus tryptophan				
1	996	48	765	18.4	18
2	1,013	27	767	21.6	145
3	987	71	758	16.0	91
Avg	999	48	763	18.8	85
	Basal diet				
1	994	48	619	32.9	168
2	1,022	36	639	34.0	271
3	979	33	569	38.5	163
Avg	999	39	609	35.1	201

[1] Each value represents the average of three dogs.
[2] Periods were of four days' duration each.

TABLE 2

Effect on nitrogen balance[1] of the omission of methionine from a casein-gelatin-tryptophan diet

Period no.[2]	Nitrogen				Gain in wt
	Intake	Fecal	Urine	Retention	
	mg/kg/day			*% of intake*	*gm*
	Basal diet				
1	817	16	452	42.7	184
2	812	52	405	43.7	124
3	808	29	534	30.3	206
Avg	812	32	464	38.9	171
	Basal diet minus methionine				
1	857	41	743	8.5	135
2	867	36	610	25.5	134
3	874	29	683	18.5	129
Avg	866	35	679	17.6	133
	Basal diet				
1	806	39	477	36.0	224
2	804	40	474	36.1	243
3	786	26	480	35.6	299
Avg	799	35	477	35.9	255

[1] Each value represents the average of three dogs.
[2] Periods were of four days' duration each.

TABLE 3

Effect of the omission of tryptophan and methionine, alone and in combination, to a casein-gelatin diet, on nitrogen balance[1]

Period no.[2]	Nitrogen				Gain in wt
	Intake	Fecal	Urine	Retention	
	mg/kg/day			*% of intake*	*gm*
	Basal diet				
1	752	46	476	30.6	278
2	755	17	496	32.1	229
3	791	39	471	35.5	228
Avg	766	34	481	32.8	245
	Basal diet minus tryptophan				
1	741	37	563	19.0	108
2	744	37	550	21.1	206
3	748	40	592	15.5	129
Avg	744	38	568	18.5	148
	Basal diet minus tryptophan minus methionine				
1	829	42	543	29.4	220
2	804	44	601	19.8	13
3	810	50	558	24.9	182
Avg	814	46	567	24.7	138
	Basal diet minus methionine				
1	775	55	559	20.8	123
2	777	39	612	16.2	234
3	786	62	661	8.0	54
Avg	779	45	611	15.8	137
	Basal diet				
1	775	47	477	32.4	560
2	766	32	458	36.0	383
3	756	41	454	34.5	328
Avg	765	40	463	34.2	424

[1] Each value represents the average of three dogs.
[2] Periods were of four days' duration each.

in nitrogen retention similar in all of the three 4-day periods, with an average of 34.2% of the nitrogen intake.

DISCUSSION

Amino acid imbalances of the type described in this paper were produced in the rat growth experiment of Harper ('59), who observed that addition of tryptophan to casein-gelatin-methionine diet corrected the imbalance as indicated by an increase in body weight gain. Using a different experimental animal and a different technique, the present results corroborate this.

The studies by Kumta et al. ('58) and Deshpande et al. ('58) with rats showed that amino acid imbalance decreased not only the percentage of ingested nitrogen retained, but also the food intake. The present results show that amino acid imbalance decreased nitrogen balance even though there was no decrease in nitrogen intake.

The variation among the three 4-day balance periods with each diet inducing imbalance of amino acids is also of interest. Figure 1 shows the behavior of the same dog in each of the experiments carried out. When either methionine or tryptophan was omitted, the response in nitrogen balance in all dogs was low in two of the three periods, usually the first and the third, and relatively high in one of the three periods, in most cases the second. This effect was more marked with the methionine omission than with the omission of tryptophan since it appears that methionine may be even more limiting than

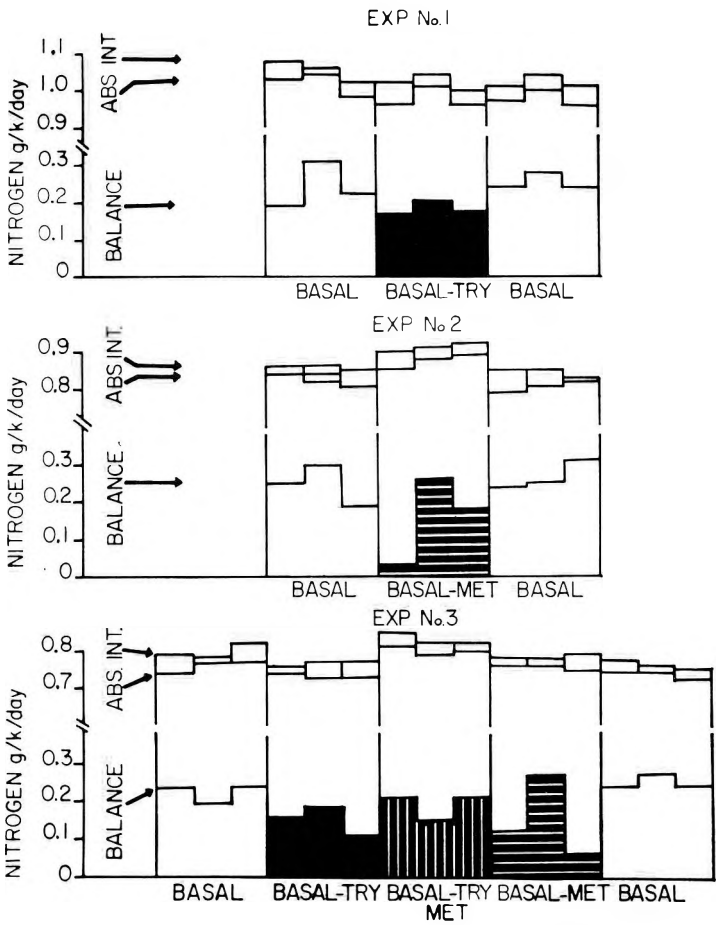

Fig. 1 Nitrogen balance of a dog fed amino acid-imbalanced diets.

tryptophan under the conditions of the experiment.

The body weight gain every 4 days appeared to follow, in general, the changes in nitrogen balance. In this case, the weight changes, when methionine was omitted, were less variable and not as low as when tryptophan was omitted. These observations were interpreted to mean that the animal adjusted to the imbalance by temporarily lowering its amino acid metabolic activities, possibly increasing the free amino acid pool to balance the pattern of absorbed amino acids by drawing amino acids from other tissues, which results in a decreased output of nitrogen. As indicated by Allison ('55) and Allison and Fitzpatrick ('60), the overall nitrogen balance is a measure of the sum of gains and losses from the various body tissues, not necessarily indicating the shifts of protein from one tissue to the other.

The results observed from the omission of the two amino acids together could be explained on the basis of a breakdown of the excess amino acids to the levels which will balance the amounts of methionine and tryptophan available. The omission of both amino acids, therefore, does not increase the severity of the amino acid imbalance beyond that already present as a result of adding 15% of gelatin to the 8% casein diet. Apparently the first limiting amino acid has a more severe effect than the two amino acids.

The above interpretations are more understandable when the proportions of methionine (M) to tryptophan (T) in the different diets tested are calculated and plotted as functions of nitrogen balance as in figure 2. The extremes of the M/T ratios resulted in a decreased retention of nitrogen. When the methionine was omitted from the basal diet, the M/T ratio is that at the lower left of the figure (2.0/1), and that at the lower right (6.6/1) represents the omission of the tryptophan supplement. The high point in the middle

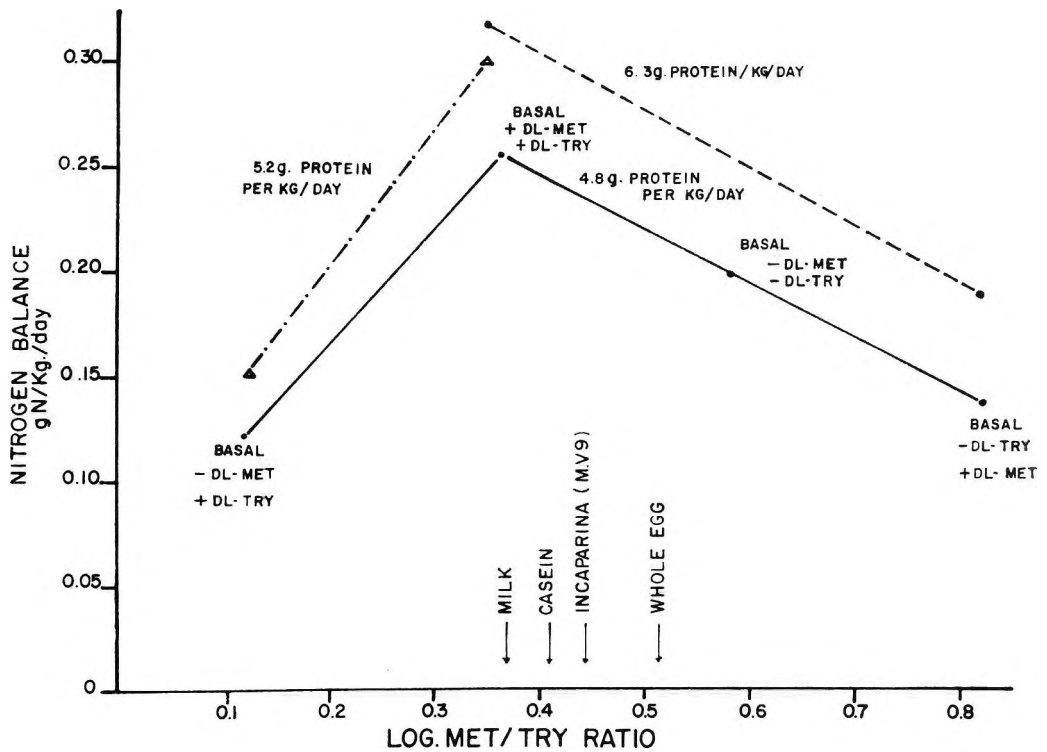

Fig. 2 Relationship between nitrogen balance and methionine-to-tryptophan ratio in dogs (casein-gelatin diet).

corresponds to the M/T ratio (3.4/1) when both amino acid supplements were present and the M/T ratio of 3.8/1, representing the omission of both, appears at mid right. The effect of the relationship between M/T appears to hold at different levels of protein intake. As would be expected, foods known to contain good quality protein have an M/T ratio in the range of those giving better nitrogen retention in the experiments described. The results indicate that the amino acid imbalances as carried out in this study result in a decrease in nitrogen balance and that it varies significantly among the three 4-day balance periods with each diet.

SUMMARY

The effect of amino acid imbalance on nitrogen retention was studied with young growing dogs, fed a basal diet containing: (in per cent) casein, 8; gelatin, 15; methionine, 0.3; and tryptophan, 0.2. Each diet was fed for three consecutive 4-day balance periods. Omitting one amino acid at a time resulted in a decrease in nitrogen balance with the most marked effect with methionine omission. The variation among the three 4-day periods was considerable when either methionine or tryptophan were omitted from the basal diet — usually because the middle periods gave relatively high nitrogen balance figures. The omission of both amino acids at the same time caused decrease in nitrogen retention over the value obtained with the basal diet. The decrease was, however, less than when only one amino acid was omitted. It was found that the methionine-to-tryptophan ratio of 2.0/1, resulting when the methionine supplement was omitted, and the methionine-to-tryptophan ratio of 6.6/1 when tryptophan was omitted, gave the lowest nitrogen retention figures. Calculation of the methionine-to-tryptophan ratio of various high quality protein foods showed that they contain approximately a 3.4/1 ratio. When the methionine-to-tryptophan ratio was 3.4/1 as in the basal diet or 3.8/1 when both amino acids were omitted, the average nitrogen retention values were higher.

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Radioactivity in Total Diet[1]

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Exposure of the human population to radiation from nuclear debris occurs predominantly from ingestion of radionuclides in food and water. Various types of information on levels of radioactivity may be required for different purposes, as for example: (a) determination of whether a given level in the diet is reached; (b) estimation of mean and maximal dietary levels as may be related to geographical considerations or dietary habits of special groups; (c) monitoring of changes in dietary levels; (d) identification for predictive capability of food components that are major contributors, and of the processes involved in transfer through the food chain; (e) development of countermeasures or remedial action; (f) provision of basis for policy decisions and recommendations by authorities; and (g) provision of information to the public. Most of the efforts have utilized analyses for specific radionuclides in human tissues (Kulp and Schulert, '61) and in various components of diet (Consumers Reports, '60, '61; HASL 111, 113, 115, 117, '61). A considerable problem is the difficulty of representative sampling without the undertaking of excessively extensive and costly survey programs (FAO, '61).

The rigor of sampling and assay required for assessment of potential hazard is related to the degree of contamination in terms of levels that are considered of significance. For example, if the levels in food were known to produce a radiation exposure of only a fraction of background exposure, then it would be wasteful of scientific effort and expenditure to attempt complete country-wide sampling and to require assays precise within a few per cent. It is necessary, then, to ascertain general levels in foodstuffs and the degree of variability among samples, so that adequate surveillance can be undertaken efficiently in accord with realistic needs.

The overall intake of radionuclides over specified time periods can perhaps be estimated with the fewest number of analyses by the use of pooled total diet samples. Although this method is efficient in terms of numbers of samples required, it gives little or no information on the mechanisms of pathway through the food chain.

In the present investigation, total diet samples were collected in January and again in May and June, 1961, from 10 to 25 major cities and analyzed for the more important radionuclides as well as for stable Ca and K. Every effort was made to have the samples represent total food and water consumption. In addition to providing an estimate of dietary levels of radioactivity, it was hoped that some measure of the sampling and assay uncertainties would be obtained from comparisons of duplicate samples from some of the cities, inter-city comparisons, and degree of agreement with results of other programs. Diet types were chosen so that any large differences as a result of dietary habits at different ages should have been observable.

METHODS

General sampling plan. As indicated in table 1, three primary studies were made in January, 1961: (a) determinations of stable Ca, Sr^{90} and Ra^{226} in total diet samples from 25 cities; (b) estimation in samples from 12 cities of Ca and Sr^{90} in milk and whole wheat constituents

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TABLE 1

Sampling plan

	Nuclides determined	No. of cities sampled	Diet-type
A January 1961[1]			
	Ca, Sr^{90}, Ra^{226}	25	middle income teenage
		3	low income teenage
		3	middle income adult
		3	infant (1 year)
	Ca, Sr^{90} in milk and wheat components	12	middle income teenage
	K, K^{40}, Cs^{137}, Ce^{144}, Pb^{210}, Pu^{239}, Zn^{65}	9	middle income teenage
		3	low income teenage
B May–June, 1961			
	Ca, Sr^{90}, Ra^{226}, K, K^{40}, Cs^{137}, Ce^{144}, Zn^{65}	10	middle income teenage
		10	low income teenage
		10	middle income adult
		10	infant (1 year)

[1] For comparative purposes, all January, 1961, studies included samples from New York, Chicago and San Francisco.

of the diet to provide direct comparisons of their relative contribution to the total diet; and (c) determination of stable K, K^{40} by calculation, Cs^{137}, Ce^{144}, Pb^{210}, Pu^{239} and Zn^{65} in total diet samples from 9 cities. Most of the studies were made on diets (for males) classified as "middle income teenage," although a few comparative studies were made on diets classified as "low income teenage," "middle income adult," and "infant," (9 to 15 months).

In the May-June, 1961, series, studies were done in 10 cities from each of which samples of the 4 types of diets were analyzed for all the nuclides mentioned above, except Pu^{239} and Pb^{210}.

Collection and preparation of samples

In each city a university or college home economist was given the responsibility for planning a typical menu, purchasing and cooking the food. The menus were chosen to represent a well-balanced diet, in the discretion of the home economist, and to include all items in amounts that typically might be consumed by normal males. The food was purchased in local markets using fresh produce when available, and frozen or canned products as the next choice. The food was prepared, cooked and seasoned as would be done in the home, using local water supplies. All inedible parts such as bones, excess fat, fruit pits and cores were removed, so that the sample represented, as nearly as possible, food as it would have been eaten. Three meals, plus snacks and drinking water, were prepared each day for a two-week period in January and a one-week period in May–June, and the edible portions collected in a large drum containing formaldehyde for preservation. At the end of the sampling period the drums were shipped to the laboratory for analysis.

Where milk and whole wheat products were analyzed separately, samples of these items were collected from the same batches of food that were used to prepare the total diet samples.

Analytical procedures

Analyses were carried out in two commercial laboratories[2] by standard radiochemical procedures (HASL, '57). The entire sample was homogenized and the aliquots were weighed, dried and ashed. The ash was dissolved in 3:1 HCl HNO_3 mixture and the solution was made up to a known volume.

Strontium-90. Calcium and strontium were repeatedly precipitated as oxalates and calcined until free from phosphates; two yttrium hydroxide scavenges were performed, the solution allowed to equili-

[2] Analyses were performed by Isotopes, Inc., Westwood, New Jersey, and Controls for Radiation, Cambridge, Massachusetts.

brate for two weeks, and the Y^{90} daughter of Sr^{90} was then separated as the hydroxide, converted to the oxalate and counted.

Cerium-144. An aliquot of solution was oxidized with sodium bromate, and extracted into methyl isobutyl ketone. The Ce was then back-extracted with hydrogen peroxide. Cerous oxalate was precipitated, and the 2.98 Mev beta particles of the Pr^{144} were counted.

Radium-226. An aliquot of solution was set aside for 4 weeks to permit full growth of Rn^{222}, which was then measured by the radon emanation technique. The same solution was then used for Pu and Zn analyses; the Pu was precipitated as $Bi(Pu)PO_4$ with the filtrate containing the Zn fraction.

Zinc-65. $ZnHg(SCN)_4$ was precipitated twice from HNO_3 solution. Two Bi_2S_3 scavengings were performed before precipitating ZnS from a NH_4AC solution. The ZnS precipitate was dissolved in HCl and two $Fe(OH)_3$-Ba CO_3 scavengings were performed before the final double precipitation of $ZnHg(SCN)_4$, which was filtered, weighed and counted.

Plutonium. Plutonium was reduced to plutonium (III) and carried on bismuth phosphate at a pH of 1.5, dissolved, and the Pu precipitated with lanthanum fluoride. The Pu was further purified by absorbing the plutonium nitrate in nitric acid on Dowex-1 anion resin and subsequently eluting it with 1 N nitric acid and hydroxylamine hydrochloride. The Pu was finally electroplated on a 4-mm diameter area of a stainless steel disk. The disk was autoradiographed and the alpha tracks counted under a microscope.

Cesium-137 and lead-210. An aliquot of original sample was heated slowly until a fairly dry mass was obtained, digested in fuming nitric acid until all organic material was consumed, then digested in concentrated perchloric acid, cooled and brought up to volume with dilute HCl. It was then neutralized with sodium hydroxide and filtered. The filtrate contained the Cs, and the Pb was contained in the precipitate.

The Cs was then precipitated as the cobaltinitrite, subsequently separated as the silicotungstate, extracted with sodium tetraphenyl boron in amyl acetate then precipitated as the chloroplatinate. The sample was weighed, mounted and counted for beta particles.

The precipitate containing the lead was dissolved in HCl. The lead was concentrated as the sulfide, converted to the iodide, and finally precipitated as lead sulfate, which was then dissolved in ammonium acetate. The Bi^{210} was permitted to equilibrate with the Pb^{210}; the Bi was precipitated as the carbonate, mounted and counted for beta particles.

In all of the above cases spikes and carriers were added at the appropriate stages and later the rates of radiochemical decay were observed to check the identity of the material being counted.

Potassium-40. Potassium-40 was determined by measuring the amount of stable K present by flame photometry, and calculating the K^{40} by using the radiation emission rates of 28.0 beta particles per second per gram of normal K and 3.45 gamma rays per second per gram of normal K.

Calcium. An aliquot of the solution was buffered with acetic acid and calcium oxalate precipitated. The precipitate was redissolved in HCl and the Ca complexed with an excess of ethylenediamine tetraacetic acid. The Ca was determined by back-titration, using calcein as indicator (Yalman et al., '59).

Reproducibility of results

Coded split samples were used for intercomparison between the two laboratories, and for estimates of reproducibility in each laboratory.

To provide some idea of uncertainties in sampling, 9 pairs of independently prepared duplicate samples were obtained from 6 cities. Each sample of each pair was planned, collected and prepared independently by a different home economist.

EXPERIMENTAL RESULTS

Table 2 presents data on analytical and sampling variability. The average differences between the same sample analyzed in the same laboratory ranged from 6 to 10% for Sr^{90}, Cs^{137} and K^{40}, whereas the differences between laboratories ranged from 24 to 33%. However, the difference values for Ra^{226} were all about 50%. It is

TABLE 2

Differences in duplicate samples from the same city and in duplicate analyses

				No. of pairs	Avg difference	
					μμc	%
1	Sr^{90}/kg	(a)	in duplicate samples from same city	9	0.4	12
		(b)	in same sample by 2 different laboratories	4	0.9	30
		(c)	in same sample by same laboratory	7	0.2	6
2	Ca/kg in duplicate samples from same city			8	0.03 (*gm*)	6
3	Ra^{226}/kg	(a)	in duplicate samples from same city	9	0.3	57
		(b)	in same sample by 2 different laboratories	4	0.2	50
		(c)	in same sample by same laboratory	4	0.3	50
4	Cs^{137}/kg	(a)	in same sample by 2 different laboratories	4	4	33
		(b)	in same sample by same laboratory	4	2	10
5	K^{40}/kg	(a)	in same sample by 2 different laboratories	4	240	24
		(b)	in same sample by same laboratory	4	70	7

particularly noted that sampling variability for Sr^{90} and Ra^{226} did not greatly exceed variability arising from analytical techniques.

Table 3 presents the data on Ca, Sr^{90} and Ra^{226} in samples of middle income teenage diets taken in January, 1961, from 25 cities, grouped by geographical region. There was general agreement in the estimated dietary consumption, Ca content and Ca intake. The average Ca intakes of 1.8 to 1.9 gm/day indicate choice of diets certainly optimum, if not above, for this element. It is not possible to generalize with respect to Sr^{90} levels between geographical regions or cities because of the many meteorological and agricultural factors that come into play. The values from the Western part of the country, with the exception of Seattle, appeared to be consistently lower than those from other regions. The highest value of Sr^{90}/gm of Ca observed was 1.8 times the country-wide mean, whereas the lowest was 0.4 of the mean value. Levels of Ra^{226} intake had a maximum-to-mean ratio of 2.8 and a minimum-to-mean ratio of 0.4 with 4 samples having counts less than the counting error.

Table 4 presents comparable data for samples collected in May–June, 1961, which show the same general pattern evident in table 3.

Table 5 presents a comparison of Sr^{90} levels in the total diet, milk components and whole wheat components from samples collected in January, 1961. Values for Sr^{90} are given in terms of micromicrocuries per gram of Ca for comparative purposes; however, values on a unit volume or weight basis can be calculated from the data presented. As expected from knowledge of the relative movement of Ca and Sr through the food chain (Comar et al., '57), the values of Sr^{90} per unit of Ca were generally higher for the total diet than for milk, with an overall ratio for diet-to-milk of 1.6 ± 0.7. In one city, San Francisco, the value deviated more than expected.

Values of Sr^{90} per unit of Ca for the whole wheat components were all higher than corresponding values for the total diet with an overall ratio of diet to whole wheat of 0.5 ± 0.2. If one assumes a value for the proportion of dietary Ca originating from dairy products, then it is possible to calculate the μμc of Sr^{90}/gm of Ca of the non-milk portion of the diet, and also the percentage contribution of milk products to the dietary Sr^{90}. This was done on the assumption that dairy products contributed 75% of the dietary Ca (National Food Situation, '61). From table 5, it can be calculated that the overall ratio of diet to non-milk components was 0.7 ± 0.4. From the last column, it can be seen that on the average the milk products contributed about 54% of the dietary Sr^{90}, while furnishing 75% of the Ca in the diet. Whole wheat products

TABLE 3

Calcium, strontium-90 and radium-226 in middle income teenage diets collected January, 1961

Region	Wt of diet	Ca content	Ca intake	Sr^{90}			Ra^{226}		
						Intake/day			Intake/day
	kg/day	*gm/kg*	*gm/day*	*μμc/kg*	*μμc/gm Ca*	*μμc*	*μμc/kg*	*μμc/gm Ca*	*μμc*
Northeast									
Boston, Mass.	4.0	0.46	1.8	4.3	9.5	17	(0.3)[1]	(0.7)	(1.2)
New York, N. Y.	2.9	0.48	1.4	3.2	7.1	9.4	0.4	0.8	1.2
Pittsburgh, Pa.	3.7	0.54	2.0	4.3	8.0	16	0.2	0.4	0.7
Average	3.6	0.49	1.8	3.9	8.2	14	0.3	0.6	1.0
North Central									
Chicago, Ill.	3.3	0.59	2.0	2.8	6.0	9.3	0.8	1.4	2.7
Des Moines, Iowa	3.4	0.54	1.8	4.1	7.6	14	(0.3)	(0.6)	(1.0)
Duluth, Minn.	3.6	0.58	2.1	6.7	12	24	1.4	2.4	5.0
Grand Forks, N. D.	3.8	0.49	1.8	3.4	7.0	13	0.3	0.7	1.1
St. Louis, Mo.	4.2	0.48	2.0	2.7	5.3	11	0.8	1.7	3.3
St. Paul, Minn.	3.5	0.50	1.8	3.2	6.4	11	0.6	1.2	2.1
Average	3.6	0.53	1.9	3.8	7.3	14	0.8	1.5	2.8
South									
Atlanta, Ga.	3.5	0.51	1.8	6.1	12	21	0.7	1.4	2.4
Austin, Texas	3.6	0.51	1.8	2.4	4.8	8.7	0.5	1.0	1.8
Coral Gables, Fla.	3.2	0.54	1.7	3.2	5.9	10	0.5	0.9	1.6
Hampton, Va.	3.7	0.54	2.0	5.4	10	20	(0.3)	(0.6)	(1.1)
Louisville, Ky.	3.8	0.56	2.2	2.6	4.8	10	0.5	0.8	1.9
Memphis, Tenn.	3.7	0.54	2.0	3.5	6.6	13	0.7	1.3	2.6
New Orleans, La.	2.9	0.44	1.3	3.6	8.6	10	0.3	0.7	0.9
Stillwater, Okla.	3.7	0.55	2.0	3.6	6.5	13	0.4	0.7	1.5
Washington, D. C.	4.1	0.46	1.9	3.7	7.9	15	0.3	0.6	1.2
Average	3.6	0.52	1.9	3.8	7.4	14	0.5	0.9	1.7
West									
Alameda, N. M.	3.5	0.51	1.8	2.4	4.7	8.4	0.3	0.6	1.0
Boulder, Colo.	3.7	0.52	1.9	2.2	4.3	8.2	0.6	1.1	2.2
Bozeman, Mont.	3.5	0.59	2.1	3.3	5.6	12	(0.3)	(0.5)	(1.1)
Los Angeles, Cal.	3.6	0.51	1.8	1.5	2.9	5.3	0.3	0.6	1.1
Nampa, Idaho	3.8	0.50	1.9	1.7	3.4	6.5	0.6	1.2	2.3
San Francisco, Cal.	3.4	0.51	1.7	2.6	4.6	8.8	0.4	0.8	1.4
Seattle, Wash.	3.9	0.54	2.1	3.9	7.1	15	0.3	0.5	1.2
Average	3.6	0.52	1.9	2.5	4.6	9.1	0.4	0.8	1.5
Maximum-to-mean ratios	1.2	1.1	1.1	2.0	1.8	1.9	2.8	2.4	2.8

[1] Data in parentheses were not used in computations since count was not larger than counting error.

TABLE 4

Calcium, strontium-90 and radium-226 in middle income teenage diets collected in May–June, 1961

Region	Wt of diet	Ca content	Ca intake	Sr^{90}			Ra^{226}		
						Intake/ day			Intake/ day
	kg/day	*gm/kg*	*gm/day*	*μμc/kg*	*μμc/gm Ca*	*μμc*	*μμc/kg*	*μμc/gm Ca*	*μμc*
Northeast									
Boston, Mass.	4.6	0.48	2.2	4.5	9.5	21	1.1	2.2	4.8
New York, N. Y.	3.4	0.51	1.7	3.6	7.1	12	0.7	1.4	2.5
Average	4.0	0.50	2.0	4.0	8.3	16	0.9	1.8	3.7
North Central									
Chicago, Ill.	3.7	0.46	1.7	2.6	5.6	9.6	0.9	2.0	3.4
Sioux Falls, Iowa	3.5	0.51	1.8	2.9	5.7	10	0.5	1.1	1.9
Average	3.6	0.48	1.7	2.8	5.6	9.8	0.7	1.5	2.6
South									
El Paso, Texas	3.5	0.48	1.7	1.8	3.8	6.3	0.5	1.1	1.8
Knoxville, Tenn.	3.5	0.45	1.6	4.7	10	16	0.5	1.1	1.7
New Orleans, La.	2.5	0.30	0.76	3.4	11	8.5	0.8	2.5	2.0
Average	3.2	0.41	1.3	3.3	8.4	10	0.6	1.6	1.8
West									
Denver, Colo.	3.0	0.57	1.7	2.8	5.0	8.4	0.7	1.2	2.0
Los Angeles, Cal.	3.0	0.44	1.3	1.9	4.2	5.7	0.5	1.2	1.6
Spokane, Wash.	3.4	0.55	1.9	3.7	6.7	13	0.5	1.0	1.8
Average	3.2	0.52	1.6	2.8	5.3	9.0	0.6	1.1	1.8
Maximum-to-mean ratios	1.3	1.2	1.4	1.5	1.6	1.9	1.6	1.7	2.0

TABLE 5

Calcium and strontium-90 in milk and whole wheat components of middle income teenage diets collected in January, 1961

Region	Calcium content			Sr^{90} Content ($\mu\mu c$/gm Ca)						Sr^{90} in diet	
	Diet	Whole milk	Whole wheat	Diet	Whole milk	Whole wheat	Non-milk components[1]	Diet/ whole milk	Diet/ whole wheat	From milk products	From whole wheat products
	gm/kg	*gm/l*	*gm/kg*							%	%
Northeast											
Boston, Mass.	0.46	1.3	0.58	9.5	6.3	15	19	1.5	0.62	49	1.4
New York, N. Y.	0.48	0.92	0.60	7.1	6.4	43	9.2	1.1	0.16	68	5.3
Average	0.47	1.1	0.59	8.3	6.4	29	14	1.3	0.39	58	3.4
North Central											
Chicago, Ill.	0.59	1.2	0.53	6.0	4.8	22	9.6	1.3	0.27	60	2.6
Grand Forks, N. D.	0.49	1.1	0.61	7.0	3.7	12	17	1.9	0.58	40	2.4
St. Louis, Mo.	0.48	1.2	0.88	5.3	4.8	12	6.8	1.1	0.45	68	3.8
Average	0.52	1.1	0.67	6.1	4.4	15	11	1.4	0.43	54	2.9
South											
Atlanta, Ga.	0.51	0.93	0.55	12	8.9	19	21	1.3	0.64	56	0.77
Austin, Texas	0.51	1.0	1.0	4.8	2.6	12	11	1.8	0.40	41	5.8
Louisville, Ky.	0.56	1.1	1.2	4.8	5.5	12	2.7	0.88	0.41	85	1.7
Washington, D. C.	0.46	1.0	0.54	7.9	4.3	28	19	1.8	0.29	40	3.5
Average	0.51	1.0	0.84	7.4	5.3	18	13	1.4	0.44	54	3.0
West											
Boulder, Colo.	0.52	1.1	1.1	4.3	2.5	6.6	9.8	1.7	0.65	44	4.5
San Francisco, Cal.	0.51	1.2	1.2	4.6	1.3	6.0	15	3.5	0.77	21	0.98
Seattle, Wash.	0.54	1.1	1.3	7.1	6.8	7.6	8.0	1.0	0.93	72	2.3
Average	0.52	1.1	1.2	5.3	3.5	6.7	11	2.1	0.78	50	2.6

[1] Calculation on assumption that 75% of dietary calcium originates from milk products.

TABLE 6

Comparison of calcium, strontium-90 and radium-226 in various diets collected in January and May–June, 1961

	Weight of diet		Calcium intake		Sr^{90}				Ra^{226}				Cities	
							Intake/day				Intake/day			
	Jan.	May–June	Jan.	May–June	Jan.	May–June	Jan.	May–June	Jan.	May–June	Jan.	May–June	January	May–June
	kg/day	*kg/day*	*gm/day*	*gm/day*	*μμc/gm Ca*		*μμc*	*μμc*	*μμc/gm Ca*		*μμc*	*μμc*		
Northeast														
Infant	1.5	1.6	1.1	1.5	5.9	7.2	6.5	10	0.7	0.5	0.7	0.8	New York	New York
Low income teenage	3.2	4.9	1.6	2.5	5.8	11	9.5	27	0.8	1.0	1.3	2.4		
Middle income teenage	2.9	4.0	1.4	2.0	7.1	8.3	9.4	16	0.8	1.8	1.2	3.7		Boston
Middle income adult	3.0	2.4	0.85	0.60	11	13	9.4	8.0	0.4	2.6	0.3	1.5		
North Central														
Infant	1.6	2.3	0.95	1.3	5.8	5.5	5.5	7.2	(0.7)[1]	0.8	(0.6)	1.1	Chicago	Chicago
Low income teenage	3.3	3.5	1.7	1.5	7.8	7.5	13	11	1.4	0.9	2.3	1.3		
Middle income teenage	3.3	3.6	2.0	1.7	6.0	5.7	9.3	9.8	1.4	1.5	2.6	2.6		Sioux Falls
Middle income adult	3.0	3.2	0.84	0.89	13	7.2	11	6.3	(1.0)	2.7	(0.9)	2.4		
South														
Infant	—[2]	1.7	—	1.1	—	8.4	—	9.8	—	1.2	—	1.2		El Paso
Low income teenage	—	3.2	—	1.8	—	7.4	—	13	—	1.2	—	2.0		Knoxville
Middle income teenage	—	3.2	—	1.3	—	8.4	—	10	—	1.6	—	1.8		
Middle income adult	—	2.6	—	0.96	—	9.5	—	9.2	—	2.4	—	2.0		New Orleans
West														
Infant	1.6	1.7	0.99	0.97	6.9	4.3	6.9	4.2	1.1	0.7	1.1	0.7		Denver
Low income teenage	3.3	3.0	1.7	1.5	5.4	5.2	9.2	7.8	1.4	1.1	2.3	1.6	San Francisco	Los Angeles
Middle income teenage	3.4	3.2	1.7	1.6	4.6	5.3	8.8	9.0	0.8	1.1	1.4	1.8		
Middle income adult	2.8	3.0	0.73	0.78	6.3	6.1	4.5	4.9	1.6	2.3	1.1	1.7		Spokane
Average by age group														
Infant	1.6	1.8	1.0	1.2	6.2	6.4	6.3	7.8	0.9	0.8	0.9	1.0		
Low income teenage	3.3	3.6	1.7	1.8	6.3	7.4	11	15	1.2	1.0	2.0	1.8		
Middle income teenage	3.2	3.4	1.7	1.6	5.9	6.9	9.2	11	1.0	1.5	1.7	2.5		
Middle income adult	3.0	2.8	0.81	0.82	10	8.7	8.3	7.1	1.0	2.4	0.7	1.9		

[1] Data in parentheses were not used in computations since count was not larger than counting error.
[2] Absence of value indicates no sample taken.

contributed only about 3% of the Sr^{90} and 1.3% of the Ca.

Table 6 presents a comparison of Ca, Sr^{90} and Ra^{226} in various types of diets collected in January and May-June of 1961. The values for January represent three cities: New York, Chicago and San Francisco. Those for May-June represent averages of two or three cities in each region as indicated in the table.

The daily Ca intakes averaged 1.1 gm for the infant diets, 1.7 gm for the teenage diets, and 0.8 gm for the adult diets. No significant differences in average Ca intake were noted between the low income and middle income teenage diets.

With respect to values of Sr^{90} per unit of Ca, it would be expected theoretically that the higher proportion of dietary Ca contributed by milk, the lower would be the Sr^{90}/Ca ratio. Simple inspection of the data in table 6 indicates that with one exception the adult values were highest and that generally the infant values were the lowest. Statistically significant differences were found between adult diets and the other diet types. No such significance was observed between low and middle income teenage diets or between infant and teenage diets.

Since both the infant and teenage diets contained a similarly high proportion of milk, there were thus only minor differences between their Sr^{90}/Ca values, whereas the values for the adult diets averaged 20 to 70% higher than the others.

Values for Ra^{226} in the May-June series are more meaningful than those of the January series, because of the larger numbers of samples. The levels of Ra^{226}/unit of Ca were about twice as high for the adult diets than for the others. In terms of daily intake, however, the teenage and adult diets gave values which were about twice those of infant diets.

Table 7 presents data by geographical regions for the estimated daily intake of various nuclides from low and middle income teenage diets. Among individual cities the K and K^{40} daily intakes ranged from 3.1 to 9.1 gm and from 2,600 to 7,800 μμc, respectively; the May-June values were generally higher, due in major part to the larger diet weights. The daily Cs^{137} intakes ranged from 16 to 112 μμc with no definite geographical or time dependency. The Ce^{144} intake averaged about 4 μμc/day with apparently aberrant values in two instances. The Pu^{239} intakes ranged from 0.03 to 0.19 μμc/day. The levels of Pb^{210} and Zn^{65} were so close to the limits of detectability that no generalizations and little meaning can be attached to the values for these randionuclides.

TABLE 7

Estimated daily intake of various nuclides from low and middle income teenage diets collected in January and May–June, 1961

	No. of cities	No. of samples	Diet wt	Intake per day						
				K	K^{40}	Cs^{137}	Ce^{144}	Pb^{210}	Pu^{239}	Zn^{65}
			kg/day	*gm*	*μμc*	*μμc*	*μμc*	*μμc*	*μμc*	*μμc*
Northeast										
January	1	2	3.1	3.7	3,000	32	0.8	2.0[1]	0.04	6.5[1]
May–June	2	4	4.4	6.5	5,600	61	3.6	—[2]	—[2]	(1.9)[3]
North Central										
January	3	4	3.6	4.2	3,400	48	4.4	4.0	0.12	(7.0)[3]
May–June	2	4	3.5	5.4	4,700	34	1.5	—[2]	—[2]	(1.8)[3]
South										
January	2	2	3.2	3.3	2,800	76	4.8	3.8[1]	0.07[1]	8.0[1]
May–June	3	6	3.2	4.7	4,000	34	2.2	—[2]	—[2]	(1.8)[3]
West										
January	3	4	3.6	4.4	3,700	48	14.4	3.3[4]	0.09	5.4[1]
May–June	3	6	3.1	4.4	3,700	31	1.7	—[2]	—[2]	(1.8)[3]
Average			3.5	4.6	3,900	46	4.2			

[1] Represents 1 sample.
[2] No determinations made.
[3] Count was not larger than counting error.
[4] Represents 2 samples.

The degree of uniformity of contamination can be judged by the observation that for Cs^{137} and Pu^{239} the ratios of maximum-to-mean values were about 2 and for Ce^{144} about 4.

Table 8 presents comparative values for nuclides of K, Cs, and Ce in various types of diets. No large differences were noted other than those resulting from variations in the level of dietary intake.

DISCUSSION

The nuclides were chosen for analysis on the basis of expected importance in contributing to the human radiation exposure. The reasons for interest in Sr^{90} are well recognized and stable Ca values are, of course, necessary for evaluation of the Sr^{90} body burden to be attained from given dietary levels of Sr^{90} (Comar et al., '57). Estimation of stable K permits calculation of radiopotassium intake which is of interest in assessment of the natural radiation exposure; it is also of convenience to express Cs^{137} levels in terms of K, although this does not imply that Cs is interrelated with K in the same way that Sr is with Ca. Values for Ra^{226} are also of considerable importance for comparison of intakes of natural and man-made radioactivity. Besides Sr^{90} and Cs^{137} the other radionuclides were anticipated to be minor contributors, but it was felt that analyses should be made. Since the food samples of this study were contaminated by events that occurred over two years previously, there were no detectable short-lived radionuclides such as I^{131} or Ba^{140}. The specific activity of C^{14} in the human population is expected to reflect that of the carbon dioxide of the atmosphere surrounding crop plants with about a year lag time (Broecker et al., '59; UNSCEAR, '60). Thus the best estimates of C^{14} body burdens may be obtained from analyses of the atmosphere rather than of diets or dietary components.

It should be emphasized that the samples did not represent diets and amounts thereof that were consumed by individuals

TABLE 8

Comparison of daily radionuclide intake in various diets collected May–June, 1961

	Wt of diet	Intake per day			
		K	K^{40}	Cs^{137}	Ce^{144}
	kg/day	*gm*	*μμc*	*μμc*	*μμc*
Northeast (Boston and New York)					
Infant	1.6	2.8	2,500	41	0.6
Low income teenage	4.9	7.7	6,600	70	4.9
Middle income teenage	4.0	5.3	4,600	52	2.3
Middle income adult	2.4	2.5	2,200	48	1.2
North Central (Chicago and Sioux Falls)					
Infant	2.3	2.7	2,300	26	0.9
Low income teenage	3.5	5.0	4,400	32	1.5
Middle income teenage	3.6	5.8	4,900	35	1.6
Middle income adult	3.2	4.3	3,600	20	1.3
South (El Paso, Knoxville, New Orleans)					
Infant	1.7	2.1	1,800	24	1.2
Low income teenage	3.2	4.8	4,100	30	2.0
Middle income teenage	3.2	4.6	3,900	37	2.4
Middle income adult	2.6	3.1	2,700	30	1.6
West (Denver, Los Angeles, Spokane)					
Infant	2.0	2.5	1,700	20	1.2
Low income teenage	3.0	4.5	3,800	34	1.9
Middle income teenage	3.2	4.2	3,600	28	1.4
Middle income adult	3.5	3.4	2,500	21	1.0
Average by age group					
Infant	1.9	2.5	2,000	27	1.0
Low income teenage	3.6	5.4	4,600	39	2.4
Middle income teenage	3.4	4.9	4,200	37	1.9
Middle income adult	3.0	3.3	2,700	29	1.3

or groups, but rather diets and amounts that home economists considered as typical for their locations. The general agreement on intakes of total diet, Ca and K among the numerous individuals who prepared the menus indicates a reasonable degree of representativeness. With respect to Sr^{90}, which is considered the most important radiocontaminant, perhaps the best parameter is the Sr^{90}/Ca ratio of the diet rather than the total Sr^{90} intake (Comar and Wasserman, '60). Thus for Sr^{90}, the relative composition of the diet is probably more important than the total food intake. The degree of uniformity of radiocontamination among samples can be gauged by the agreement in Sr^{90} values from pairs of samples from the same city and in samples from among cities.

The values for micromicrocuries of Sr^{90}/gm of Ca are in general agreement with those obtained by others from analysis and summation of individual dietary components. Values for adult diets during November, 1960, to February, 1961, from New York, Chicago and San Francisco were reported as 9.3, 7.9 and 3.6 (HASL 113, 115, '61); these compare with 11.3, 13.2 and 6.3 as reported here for adult diets, and 6.5, 6.9 and 5.0 for teenage diets. For the period April-June, 1961, adult diets were reported as 11.0 and 9.1 for New York and Chicago (HASL 115, 117, '61), as compared with 10.5 and 7.4 observed in this study.

Attention has always been focussed on milk because it is convenient for large-scale sampling programs, serves as a primary source of Ca in the diet, and may comprise the greater part of the dietary intake for infants and young children. From knowledge of the comparative utilization of Ca and Sr, it can be postulated that if the plant foods consumed by man and the dairy animal contain the same Sr^{90}/Ca ratio, then milk would have a Sr^{90}/Ca value of one-tenth that of plant sources of Ca (Comar et al., '57). Because of such factors as surface contamination and contrasting rooting habits of grasses and vegetables, this theoretical ratio will not be attained until the fallout Sr^{90} becomes uniformly distributed in all of the Ca that is available to plants. The data of table 5 permit several inferences: (a) milk Ca contained about one-half the Sr^{90} contamination of other dietary sources of Ca; (b) if milk had been completely eliminated from the diet, the Sr^{90}/Ca intake would have been increased by a factor of about 1.8; (c) dairy products contributed 54% of the dietary Sr^{90} assuming that they supplied 75% of the dietary Ca; (d) the ratio of diet-to-whole milk is sufficiently constant for given periods of time that whole milk values can be helpful in estimating total diet values on a nationwide basis with uncertainties acceptable for purposes of hazard evaluations. This confirms similar observations in 1959 (Michelson, '61).

The milk values in table 5 agree reasonably well with similar values estimated by others during the first quarter of 1961. For example, values from other sampling programs for New York City ranged from 5.3 to 8.3 μμc of Sr^{90}/gm of Ca, compared with 6.4 reported here; for Chicago, 3.4 to 7.0 compared with 4.8; for San Francisco, 1.7 to 4.0 compared with 1.3 as indicated in table 5 (HASL 113, 115, '61; RHD, '61).

For Ra^{226} it is not known whether the most important parameter is the daily intake, intake per unit weight of diet, or intake per unit of Ca. For present purposes, comparisons are made in terms of daily intake. Early data on the levels of Ra^{226} in food and water have been reviewed by Stehney ('60). Limited analyses of foods in the United States indicated a daily intake of the order of 1 μμc, most of which came from food rather than water with some prominent exceptions. Drinking water for most large cities has been shown to contain of the order of 0.1 μμc of Ra^{226}/liter (Hursh, '54). A daily intake of 3 μμc of Ra^{226} has been estimated for a "standard man" from analyses of foods in Germany (Muth et al., '60). From analyses of excretion rates, Stehney and Lucas ('56) have estimated an intake of about 1.6 μμc/day for 7 individuals in Chicago. Values from summation of individual components in adult diets in New York, Chicago and San Francisco have been reported as 2.4, 1.9 and 1.7 μμc/day, respectively, and for infant diets in New York City as 0.6 (HASL 113, '61). Teenage total diet sam-

ples from 5 U.S. cities had an average intake of 3 μμc/day (Michelson, '61). Thus there is general agreement with the values found in this study: namely, an average intake of about 2 μμc of Ra^{226}/day from teenage and adult diets, and about 1 μμc/day from infant diets as reported in table 6.

For comparison, the relative daily intakes of the various radionuclides were as follows: (rounded averages expressed in micromicrocuries in decreasing order) K^{40}, 4,000; Cs^{137}, 50; Sr^{90}, 10; Ce^{144}, 4; Pb^{210}, 4; Ra^{226}, 2; Pu^{239}, 0.1. Many other factors, of course, would have to be taken into account in an assessment of relative radiation dosage and potential harm from these radionuclides.

SUMMARY

1. Total diet samples collected in 1961 from 10 to 25 major cities were analyzed for radionuclides arising from nuclear debris (Sr^{90}, Cs^{137}, Ce^{144}, Pu^{239}, Zn^{65}), for natural radioactivity (Ra^{226}, Pb^{210}, K^{40}), and for the stable nuclides of Ca and K. The variability arising from collection of samples was not greater than that due to analytical procedures.

2. Levels of Sr^{90} in the total diet ranged from 2 to 15 μμc/gm of Ca. Values in the West, with the exception of the Far Northwest, tended to be lower, but no marked differences were observed among other geographical regions. The Sr^{90}/gm Ca values for adult diets, presumably because of their lower milk content, were higher than for infant and teenage diets.

3. The values of Sr^{90}/ gm of Ca for the total diet were about 1.6 times those for the milk contained in the diet. It was calculated that 54% of the total Sr^{90} intake came from dairy products, on the assumption that 75% of the Ca originated from this source.

4. The daily intake of Ra^{226} was estimated to be about 2 μμc in teenage and adult diets, and about 1 μμc in infant diets.

5. Average daily intakes of the various radionuclides expressed in micromicrocuries, were as follows: K^{40}, 4,000; Cs^{137}, 50; Sr^{90}, 10; Ce^{144}, 4; Pb^{210}, 4; Ra^{226}, 2; Pu^{239}, 0.1.

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Effect of Diet Upon the *in vitro* Metabolism of Rat Epididymal Adipose Tissue[1]

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It has long been known that the composition of adipose tissue is influenced to a greater or lesser degree by the fatty acid composition of the dietary fat. Under some conditions there may be a remarkable similarity between the two (Hegsted et al., '60). On the other hand, the composition of adipose tissue does not duplicate that of the dietary fat. This selectivity could be explained in several ways. First, some of the dietary fatty acids may be more readily metabolized before reaching the adipose tissue (Kirschner and Harris, '61; Bloom et al., '51); also, certain fatty acids may be either less readily incorporated into adipose tissue or more readily mobilized;[3] and finally, dietary fatty acids may be diluted by those acids synthesized *de novo* in the body. One could also assume that the more fat there is in the diet, the less is the need for lipogenesis (Hill et al., '60).

It is also recognized that fatty acids of adipose tissue are released and transported to other tissues to be utilized for energy purposes. Evidence in support of this knowledge was reported by Gordon and Cherkes ('56), Dole ('56) and Gordon ('57). It has also been shown that the *in vitro* release of fatty acids is influenced by a number of hormones (Schotz et al., '59, Leboeuf et al., '59; Engel and White, '60).

This report is a study to determine whether diet composition has pronounced effects upon *in vitro* lipogenesis and release of fatty acids from epididymal adipose tissue. Answers to several questions were sought: (a) is there a quantitative difference in lipogenic acivity and in the fatty acids released *in vitro* from fat pads of rats previously fed various kinds and amounts of dietary fats; (b) what acids are released *in vitro* when adipose tissue is under the influence of epinephrine; and (c) do dietary fats quantitatively affect the level of adipose tissue lipase?

EXPERIMENTAL

The design of the first experiment, data of table 1, consisted of 6 groups of male

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[3] Rizack, M. A. 1960 Thesis, Rockefeller Institute, New York, as cited by B. Jeanrenaud, Metabolism, *10:* 535, 1961.

TABLE 1

Effect of diet on lipogenesis in rat epididymal adipose as measured by the manometric determination of carbon dioxide output

Type of diet fed	Brief description	Carbon dioxide
		μliters/100 mg tissue/hour
Purified	glucose + 5% coconut oil	71[1] (56–81)[2]
Purified	starch + 5% coconut oil	62 (41–85)
Purified	glucose + 25% coconut oil	14 (9–17)
Purified	starch + 25% coconut oil	17 (10–24)
Natural ration	cereals + about 1% fat	40 (30–70)
Commercial laboratory chow[3]	about 6% fat	37 (21–56)

[1] Mean value of at least eight determinations.
[2] Range of values.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

rats, 5 rats/group, and each rat weighing 60 to 70 gm. All rats were obtained from the Charles River Breeding Laboratory, Boston. The purified diets containing the smaller amount of fat consisted of: (in per cent) glucose or starch, 67; casein, 20; cystine, 0.1; salt mixture (Hegsted et al., '41), 4; and fat, 5. Fat-soluble vitamins were provided by a mixture of vitamin A acetate, vitamin D and α-tocopherol; water-soluble vitamins were provided as previously described (Nakamura et al., '60). In the diets containing larger amounts of fat, isocaloric substitution of fat for carbohydrate was made on the basis of 3.4 Cal./gm of carbohydrate and 9.0 Cal./gm of fat. The natural ration consisted of: (in per cent) linseed oil meal, 15; yellow corn meal, 21; ground malted barley, 10; red dog wheat, 22; oat flour, 15; dried skim milk, 12; soluble blood flour, 3; salt, 1; steamed bone meal, 1; and vitamin A, 100 units/100 gm. An additional group received commercial laboratory chow.[4] All animals were fed diets ad libitum for 21 days.

After the animals were fed the diet for 21 days, two rats from each group were decapitated on the first day, two more from each group on the second day, and the fifth rat on the third day. Duplicate pieces of tissue from the tip of an epididymal fat pad were excised, weighed (approximately 244 mg), and each piece placed in one of two Warburg flasks containing 3.1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit et al., '51) and 0.02 M glucose. Six-tenths unit of insulin was contained in the flask side arm. Flask and contents were kept at room temperature prior to commencement of the experiment, then placed in the 37° water bath, gassed with a 5% carbon dioxide-95% air mixture for 10 minutes while shaking, temperature equilibrated for an additional 5 minutes, and the stopcocks closed. Following a 20-minute control period, the insulin was tipped into the main compartment of the flask and gas pressure changes noted at 15-minute intervals for one hour. The results are expressed as microliters of carbon dioxide evolved per 100 mg of fresh tissue per hour (Ball et al., '59).

The second experiment, data of table 2, consisted of young male rats weighing 40 to 50 gm which were divided into 18 groups of 4 animals each. The composition of the purified diets was the same as stated for experiment 1 and the animals were allowed these diets ad libitum for 64 days.

At the time of sacrifice it was possible to manage only 9 animals per day. On the first day one animal from each of the first 9 groups was killed and an animal from each of the remaining groups on the second day. On the third day the procedure was reversed, starting with an animal from group 18 and so forth. While this was not random, it avoided a consistent pattern in killing animals which might have influenced the results. Rats were decapitated and tips of each epididymal pad were removed, weighed (approximately 200 mg) and placed in duplicate Warburg flasks. The manometric procedure followed was the same as described for experiment 1. Another piece of epididymal adipose tissue was excised (approximately 500 mg) and placed in a previously prepared beaker containing 9 ml of Krebs-Ringer bicarbonate buffer containing 3% albumin.[5] Approximately 30 minutes were required to kill 9 animals and both flasks and beakers remained at room temperature during this time. Beakers were placed in the 37°C water bath of the Dubnoff Metabolic Shaker, and gassed with 5% carbon dioxide — 95% air for 5 minutes while shaking at 60 to 70 cycles/minute. Then 3 ml each of 0.5% glucose and 0.91 mmoles of epinephrine bitartrate in buffer-albumin solution were added to each beaker. Aliquots were taken at zero time (immediately following the addition of glucose and epinephrine) and following the 4-hour incubation period in order to determine the total fatty acids released. Total fatty acids released were determined by the method of Dole and Meinertz ('60).

The epididymal adipose tissue was removed from the beaker with forceps and rinsed with distilled water at the end of the experiment. Washings were combined with the incubation medium and sufficient 3.5 N sodium hydroxide added to raise the pH to 8.6 to 9.6. The alkaline mixture was

[4] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[5] Bovine Albumin Fraction V, Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Effect of kind and amount of dietary fat on lipogenesis and fatty acid release from rat epididymal adipose tissue

Fat fed	Level	Carbon dioxide	Rate of fatty acid release[1]
	% total Cal.	*μliters/100 mg tissue/hour*	*%*
Safflower	12	9.8[2]	1.08[3]
	24	9.7	1.14
	48	3.0	1.92
Corn	12	35.0	1.51
	24	8.5	1.94
	48	4.5	1.32
Olive	12	22.3	1.72
	24	6.5	1.28
	48	1.7	0.81
Hydrogenated cottonseed	12	16.4	1.71
	24	11.9	1.38
	48	4.3	1.36
Butter	12	15.5	1.43
	24	11.1	1.54
	48	7.9	1.08
Coconut	12	30.0	1.76
	24	35.1	1.49
	48	5.6	1.59

[1] Milliequivalents of fatty acid released/100 mg tissue/4 hours.
[2] Value reported is the mean of at least 8 determinations.
[3] Mean value for 4 rats.

extracted with petroleum ether (30 to 60° boiling fraction), the ether discarded, and the extracted solution acidified to pH 2.5 with 5 N sulfuric acid. Free fatty acids were extracted from the acidified solution with petroleum ether. The petroleum ether extract containing the free fatty acids was washed with distilled water and dried over anhydrous sodium sulfate. The ether-sulfate mixture was filtered prior to transmethylating the fatty acids by the method of Stoffel et al. ('59). Fatty acids were identified as the methyl esters by gas-liquid chromatography (table 3). The operating conditions of the gas chromatograph have been described (Hegsted et al., '60).

The third experiment (data of table 4) was to determine whether dietary fat had an effect on the *in vitro* lipase activity of epididymal adipose tissue. Young male rats weighing about 45 gm were maintained in individual cages, fed the commercial laboratory chow for two days, then divided into 4 groups of 8 animals per group. One group was fed the commercial laboratory chow, the second a purified diet containing 5% of coconut oil, a third group was fed the same purified diet but with 20% of coconut oil, and the last group fed a purified diet containing 5% of safflower oil. The compositions of the purified diets were nearly isocaloric and the same as stated for experiment 1. All diets were allowed during the 4-week experimental period.

The experiment was started one week after feeding diets to all groups of rats. One rat per group was anesthetized with pentobarbital sodium[6] (6 mg/100 gm body weight) for 30 minutes prior to removal of both epididymal fat pads. A second rat from each group was killed two days later for a total of two rats per group per week. This procedure was continued for 4 consecutive weeks. The fat pads were excised, weighed and a modified method of Rizack ('61) used to determine the lipolytic activity. Three volumes of cold 0.25 M sucrose were added to the minced tissue followed by homogenization at room temperature until creamy smooth. The homogenate was centrifuged at 10,000 $\times g$ for 10 minutes at 2°C. Three phases resulted by centrifugation; the top semisolid fat layer,

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

TABLE 3

The in vitro release of fatty acids from rat epididymal adipose tissue under the influence of epinephrine

Fatty acid	Source of fatty acid	Kind and amount of fat in rat diet																		Conclusion
		Olive, %			Safflower, %			Coconut, %			Corn, %			Hydrogenated fat,[2] %			Butter, %			
		5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	
		% Composition																		
16:0[1]	Epididymal adipose tissue, *in vitro*	25	18	11	23	22	15	23	24	21	20	20	18	31	20	14	31	34	29	Fatty acid released proportional to amount in tissue
		24	16	14	23	19	12	22	31	35	24	22	18	28	23	14	28	32	32	
16:1	Epididymal adipose, tissue, *in vitro*	9	4	2	9	5	2	14	12	6	7	4	2	11	6	3	14	11	7	Fatty acid released in larger amounts than found in tissue
		22	8	3	17	12	1	22	22	21	14	11	6	16	6	4	21	23	15	
18:1	Epididymal adipose, tissue, *in vitro*	59	72	80	28	20	17	42	36	35	40	32	32	47	63	66	46	43	46	Fatty acid released in lesser amounts than found in tissue
		42	63	64	20	13	14	41	26	28	27	21	20	37	44	58	56	36	38	
18:2	Epididymal adipose, tissue, *in vitro*	3	3	6	36	49	63	2	3	3	29	41	45	5	6	11	1	2	2	Fatty acid released proportional to amount found in tissue
		5	8	10	37	52	69	6	6	7	34	41	51	11	12	18	3	3	3	

[1] First figure indicates the number of carbon atoms; the second, the number of double bonds.
[2] Spry, Lever Brothers, New York.

TABLE 4

The in vitro lipolytic activity of an extract of epididymal adipose tissue from rats fed various diets

	Diet fed	Lipolytic activity[1]	P value between groups
(A)	Commercial laboratory chow[2]	622 ± 218[3]	
(B)	Purified diet containing 5% coconut oil	462 ± 224	A-B $P < 0.40$ D-B $P < 0.50$
(C)	Purified diet containing 20% coconut oil	393 ± 179	A-C $P < 0.05$ B-C $P > 0.50$ D-C $P < 0.05$
(D)	Purified diet containing 5% safflower oil	602 ± 170	A-D $P < 0.10$

[1] Milliequivalents of fatty acid released per hour.
[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[3] Mean and standard deviation for 8 determinations.

the clear infranatant, and a pellet on the bottom of the tube. Two-tenths milliliter of infranatant was pipetted into test tubes containing 0.1 ml of a commercial cottonseed oil emulsion;[7] 0.2 ml of 0.06 M potassium phosphate buffer, pH 6.8; 0.5 ml of 20% albumin dissolved in phosphate buffer; and sufficient distilled water to make a final volume of 2.0 ml. All tubes were incubated at 37°C for one hour and the experiment terminated by addition of the extraction mixture of Dole and Meinertz ('60) which was used for the microdetermination of fatty acids. Zero time controls for initial fatty acid content consists of unincubated samples of reagents and infranatant — sucrose-extract of epididymal adipose tissue.

RESULTS

Table 1 reports the effects of several diets on *in vitro* lipogenesis in rat epididymal adipose tissue as measured by the manometric technique. Epididymal adipose tissues from rats fed the same diet often show considerable differences in activity (range of values shown in parentheses). The results demonstrate that high-fat diets depress fat synthesis *in vitro* and it appeared to make no difference whether the dietary carbohydrate was glucose or starch. The two crude rations as compared with the purified diets resulted in considerably less lipogenic activity than would have been expected if the fat content alone was the determining factor. Apparently some factor other than the fat content is also involved.

In the next experiment, table 2, the high-fat diets depressed fat synthesis *in vitro* regardless of the type of dietary fat. On the other hand, considerable differences were noted in results due to the various fats. The most active lipogenic tissues were from animals fed coconut oil. Increasing the level of this fat from 12 to 24% of total calories did not depress lipogenesis. The least active tissues were from rats fed safflower oil. It is tempting to try and relate lipogenic effects to the degree of unsaturation of dietary fats. Safflower is the most unsaturated oil and contains approximately 70% of linoleic acid. Corn oil is next, followed by olive oil, hydrogenated cottonseed oil, butter and then coconut oil which is about 90% saturated. However, certain discrepancies were evident. Tissue from the group fed corn oil was more active than expected at the low dietary level when compared with olive oil; tissue from butter-fed animals was less active than expected.

A sample of each epididymal pad was obtained for fatty acid analysis by gas chromatography. Although there were marked differences in composition,[8] which in general were related to the composition of the fat fed, we were unable to explain lipogenic differences in terms of the fatty acid composition of the adipose tissue.

Table 5 presents the analysis of variance of the data for lipogenesis in table 2. The most important variable is the level of fat

[7] Lipomul I.V., Upjohn Company, Kalamazoo, Michigan.
[8] Di Giorgio, J., R. Bonanno and D. M. Hegsted, unpublished data.

TABLE 5

Analysis of variance of CO_2 evolution data

Source of variation	Degree of freedom	Σ^2	Mean square	F	P
Kind of fat	5	3977.07	795.41	4.63	< 0.01
Level of fat	2	6956.37	3478.19	20.24	< 0.001
Interaction	10	4352.15	435.22	2.53	$> 0.05 < 0.01$
Within groups	126	9988.69	79.28		
Within animals	72	721.53	10.02		
Between animals	54	9267.16	171.61[1]		

[1] This value used in the denominator in calculating *F* values for the major effects since it is the largest of the error terms.

fed. However, the kind of fat has, likewise, a highly significant effect. Somewhat unexpectedly, the interaction term reached only a low level of significance. This would suggest that the effects of the kind and of the amount of dietary fats are independent of each other to a considerable extent.

The amounts of fatty acid released from epididymal adipose tissue when under the influence of epinephrine *in vitro* is likewise shown in table 2. The mean rate of release under these experimental conditions was 1.47 ± 0.316 μEq./100 mg of fresh tissue/4 hours of incubation. Variance analysis revealed no significant effect of the different dietary fats. These values were obtained with animals decapitated without the prior administration of pentobarbital sodium.

The above result raised two questions: was the 4-hour incubation too long, thereby masking any real differences which may have been apparent during the early part of the experiment or were there indeed no differences in tissue lipolytic activity? Table 4 summarizes data from an experiment performed to answer the latter question. Inspection of the data for lipolytic activities suggests that although differences cannot be excluded there is little evidence of a dietary effect.

The composition of the fatty acids released *in vitro* was dependent upon the composition of the parent fat pad. As shown in table 3, the percentage of palmitic and linoleic acids in the free fatty acids released during incubation of tissue under the influence of epinephrine was essentially proportional to the amount of fatty acids in the tissue. The proportion of oleic acid in the free fatty acids was slightly less than in the tissue, perhaps significantly so. The regression coefficients are significantly less than one at the 5% level but not at the 1% level of significance. In contrast, palmitoleic acid was released in relatively large amounts as compared with the tissue content. The regression coefficient indicates that there was approximately 1.5 times as much in the free fatty acids as in the tissue. This is greater than one at a high level of statistical significance. Thus, it appears that the palmitoleic acid or a portion of it is more labile under these conditions than the remainder of the fatty acids.

DISCUSSION

The data of tables 1 and 2, wherein high fat diets cause a depressed fat synthesis *in vitro*, agree with results of Hausberger and Milstein ('55). They reported that epididymal adipose tissue incubated with uniformly labeled glucose as substrate showed a complete inability for fatty acid synthesis when the rats had been previously fed a 60% fat diet; a 35% fat diet allowed only slight lipogenesis. However, utilization of glucose for lipogenesis increased to a significant level with a 13% fat diet and was increased to the highest rate by feeding a practically fat-free diet (0.8%). Hill et al. ('58) have demonstrated that hepatic lipogenesis is depressed by a high fat intake, even when the diet contained a high percentage of carbohydrate.

It is clear, however, from the results reported here that lipogenesis in such studies is not solely dependent upon the amount of fat in the diet. The kind of dietary fat has a significant effect. In gen-

eral the feeding of the more unsaturated oils depressed lipogenesis in the fat pad but the correlation with over-all degree of unsaturation or the content of specific fatty acids was poor and does not appear to offer adequate explanation. Similarly, unpublished data on the fatty acid composition of the adipose tissue does not appear to explain the differences in activity observed.

Studies with crude diets indicate that the lipogenic activity of the epididymal fat pads from such animals are considerably less active than those obtained from animals receiving purified diets with comparable levels of fat. Substitution of starch for glucose in the purified diet did not decrease the activity. Thus, there appear to be unknown dietary factors that determine the lipogenic activity.

A large number of variables may influence the results obtained in studies of the kind reported in table 2. Fatty acid release is enhanced by fasting. Gordon and Cherkes ('56) reported little release of fatty acids from tissues of fed rats even though the medium contained no fatty acids. The animals used here had food available up to the time of sacrifice. The rate of release is dependent upon the time of incubation. Lynn et al. ('60) and Hagen ('61) found that accumulation of fatty acids decreased after one hour. Thus, longer incubation periods might obscure differences evident under shorter incubation times. The possibility of interchange between fatty acids released and tissue fatty acids may be increased by long incubation. The animals in the second experiment were decapitated, whereas those in the third (table 4) received pentobarbital sodium 30 minutes before sacrifice. It has been shown that pentobarbital anesthesia with the simultaneous injection of a ganglionic blocking agent decreases lipolytic activity (Rizack, '61). We have found[9] that anesthetizing with pentobarbital sodium alone caused a significant decrease in activity as compared with that obtained with animals decapitated. Since adipose tissue can store epinephrine (Paoletti et al., '61) these effects are presumably explained by the excitation of the animal resulting in either storage of catecholamines or activation of the lipolytic system. Finally, the composition of the substrate utilized to study lipolysis may be important since lipases have some degree of specificity.[10] In animals fed different fats, the substrate is presumably changed. It is not known whether data obtained with unnatural substrates, such as the cottonseed oil emulsion used in some of the experiments reported, or the coconut oil emulsion used by Rizack ('61), are entirely relevant. It should also be pointed out that the data reported in table 4 were obtained from the infranatant fraction although some activity is left in the particulate fraction. With these large numbers of variables, the question of dietary effects upon lipase activity must remain problematical although the evidence obtained here suggests that they are probably minimal.

Regression equations were calculated relating the percentage of each fatty acid in the fatty acids released, to the percentage of the same fatty acid in the adipose tissue fatty acids. With two exceptions the slope of the regression line was not significantly different from 1.0, indicating a rate of release proportional to the amount of fatty acid present in the tissue. On the other hand, the slope of the regression line for oleic acid was 0.82, significantly different from 1.0, indicating that oleic acid was relatively resistant to release. Palmitoleic acid was released in proportions greater than those in the tissue, the slope of the regression line being 1.5. From the values for 4 of the fatty acids given in table 3, the amounts of palmitoleic acid tend to be higher in the tissues of animals that are also higher in palmitic acid. Since none of the dietary fats contained appreciable amounts of palmitoleic acid, this fatty acid must be synthesized in the animal and presumably originates from palmitic acid which is either synthesized or deposited from the dietary fat.

Palmitoleic acid may, therefore, occupy some special position within the triglyceride which makes it more vulnerable to tissue lipase or hydrolysis. The opposite appears to be true of oleic acid although

[9] See footnote 8.

[10] Washburn, L., R. Brown and W. S. Lynn 1960 Liberation and stimulation of adipose tissue lipases. Federation Proc., *19:* 224 (abstract).

the amounts originating from the diet and from synthesis are unknown. In view of the large amounts of oleic acid in tissues, as compared with palmitoleic acid, the differences in the total amounts of fatty acids released are greater for oleic acid than palmitoleic acid although the percentage difference is greater for the latter.

SUMMARY

Diet composition has a pronounced effect upon the lipogenic and lipolytic abilities of epididymal adipose tissue *in vitro*. The level of dietary fat is an important variable in that high fat diets depress *in vitro* lipogenesis. On the other hand, this is not a uniform characteristic of all fats since their effect is variable, depending upon the kind of dietary fat. However, the lipogenic activities of tissues from rats fed two crude diets were less when compared with tissues from rats fed purified diets of a similar fat content. Hence, some factor other than dietary fat is presumably involved in these instances.

Rats were fed crude and purified diets for several weeks in order to study their effects on the *in vitro* release of fatty acids from rat epididymal tissue. The purified diets were isocaloric and differed only in the kind and amount of dietary fat. It was found that the total quantities of fatty acids released during a 4-hour incubation period of epididymal adipose tissue from the various groups of rats were not significantly different. However, the adipose tissue fatty acids were released in proportions different from those initially present in the parent tissues. Palmitoleic was released in relatively greater and oleic acid in lesser amounts than expected from the tissue composition.

There was no difference in the extractable lipolytic activity of epididymal adipose tissue from rats fed several dietary fats.

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The Nature of the Antithyrotoxic Effect of Liver Residue[1]

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Numerous laboratories have demonstrated that the hot water-insoluble, heat-coagulated, fraction of hog or beef liver (liver residue) possesses antithyrotoxic properties. This antithyrotoxic effect has usually been demonstrated by feeding some form of thyroxine (usually desiccated thyroid or iodinated casein) to weanling rats and preventing the growth inhibition that accompanies the thyrotoxicosis by adding liver residue to the otherwise highly purified diet. The pertinent literature was cited recently in an excellent series of papers by Overby et al. ('59a,b,c), Overby and Fredrickson ('60a,b, '61) and Dryden et al. ('60). This antithyrotoxic effect has generally been attributed to the presence of an unidentified growth factor in the liver residue, as it was believed that the thyrotoxic state exhausted the limited supplies of this factor otherwise available to the rat, and thereby allowed its demonstration by the criterion of growth. However, this explanation was recently questioned by Overby and Fredrickson ('60b) when they could not demonstrate an exhaustion of this factor from the liver or muscle of hogs made thyrotoxic.

In previous studies we (Westerfeld and Richert, '52) noted incidentally that the color of the hyperthyroid rat liver was usually much darker than was true of the euthyroid rat liver. The high speed supernate of a sucrose homogenate of normal rat liver exhibited a characteristic iron-porphyrin spectrum similar to hemoglobin. Based upon the optical density of the 410-mμ peak, the liver from the hyperthyroid rat contained 50% more iron porphyrin (not further identified) per unit weight than was present in the normal control rat liver. When the thyrotoxic growth inhibition was effectively reversed by including a combination of liver residue, cod liver oil and egg yolk in the diet, the gross color of the liver as well as the optical density at 410 mμ returned to normal. This suggested that the growth response obtained from the incorporation of these substances into the diet was actually due to a reversal of the hyperthyroid state itself rather than to a vitamin-like growth factor.

A differentiation between these two possibilities was made by determining the effect of these dietary additions on the metabolic rate of the thyroid-fed rat. The addition of a vitamin-like growth factor to the diet would not be expected to reduce the metabolic rate even though it might possibly restore a normal growth rate. The results showed clearly that the growth response exhibited by liver residue was due to a prevention of the hyperthyroid state, and the liver residue therefore contained an unknown "antithyroid" factor. The magnitude of the effect of liver residue on the metabolic rate of the thyroid-fed rat depended upon the relative amounts of the two factors added to the diet. With a relatively large amount of iodinated casein[2] (e.g., 0.35%) and a modest amount of liver residue (e.g., 10%) in the diet the decrease in metabolic rate was not particularly large. However, the effect was clearly and dramatically evident when the diet contained less iodinated casein or more liver residue, or both.

METHODS[3]

Weanling male rats of the Holtzman strain, weighing approximately 50 gm,

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[2] Protamone.
[3] Protamone (iodinated casein) was obtained from the Cerophyl Laboratories, Kansas City, Missouri, and contained 1% thyroxine equivalent. Phillips and Hart salts, and the casein were obtained from Nutritional Biochemicals Corporation, Cleveland. A vitamin D_3 preparation containing 200,000 USP units/gm was obtained from Charles Bowman Company, Holland, Michigan.

were fed the various diets for 4 weeks. The basal diet was similar to one used by Overby et al. ('59a), except for the omission of inert material, and consisted of the following: (in per cent) casein, 30; cottonseed oil, 10;[4] salt mixture (Phillips and Hart, '35), 4; sucrose, 56; choline chloride, 0.1; and inositol, 0.05. Vitamins were added as follows: (in mg/kg of diet) riboflavin, 25; thiamine·HCl, 50; niacin, 100; pyridoxine·HCl, 15; folic acid, 5; Ca pantothenate, 200; *p*-aminobenzoic acid 100; menadione, 1; mixed tocopherols, 600; vitamin B_{12}, 0.1; vitamin A, 40; vitamin D preparation, 20; and biotin, 0.5.

Extracted liver residue (LR) was usually substituted for an equivalent amount of casein to keep the total protein constant at 30%. Unless otherwise noted, the liver residue used in these experiments was prepared from beef liver.[5] It was extracted locally in a large scale soxhlet apparatus, first with hot 95% alcohol and then with hot chloroform, to remove the lipids.

The metabolic rate (MR) was determined between 10 AM and 4 PM with an apparatus similar to the one described by Robbie ('48); the exponent 0.73 was used to convert body weight to square meters of body surface. Five rats from each diet group were used for this purpose, and the MR was determined three times on different days for each rat. Such determinations were not true basal measurements since the rats were not fasted, and small movements were possible. However, the same relative differences in metabolic rates between rats fed the basal diet ± 0.1% of iodinated casein ± 5 or 10% of liver residue were obtained when the rats were fasted or fed overnight and no advantage resulted from the additional manipulation of fasting. The small size of the respiration chamber and a 5-minute preliminary acclimation period minimized the physical activity of the rats during the measurement, but movement probably increased the oxygen consumption relatively more with the hyperthyroid than with the normal rats, and thereby exaggerated the differences between them. The values obtained are called metabolic rates (MR) instead of basal metabolic rates, and were reproduced satisfactorily from day to day. Differences between diets were also reproduced satisfactorily in independent experiments, but the absolute values varied somewhat and were controlled by including: (1) a nonthyroid basal diet group; (2) an unprotected iodinated casein diet group; and (3) an iodinated casein plus standard liver residue diet group with each experiment.

RESULTS

Table 1 shows the protective effect exerted by LR on MR and tissue cytochrome c levels (as determined by the method of Rosenthal and Drabkin, ['54]) as well as on the growth of hyperthyroid rats. Groups of 8 rats each were fed the indicated diets containing 0.35% of iodinated casein for 4

[4] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.
[5] Prepared by Armour and Company, Chicago.

TABLE 1

Inhibition of the hyperthyroid effects by liver residue

Diet composition				4-Week body wt	Metabolic rate	Heart		Liver
Casein	Liver residue	Cod liver oil	Egg yolk			Weight	Cytochrome c	Cytochrome c
%	%	%	%	*gm*	*l* $O_2/m^2/hr$	*mg/gm body wt*	*μg/gm wet wt*	*μg/gm wet wt*
30[1]	—	–	—	230	8.4	4.0	266	78
30	—	–	—	130[2]	15.7	8.1	353	247
20	10	–	—	195	12.7	6.5	332	194
15	15	–	—	205	9.9	5.5	303	169
30	—	2	—	140[2]	15.3	8.8	346	265
15	15	2	10	225	9.3	5.3	288	139

[1] Basal diet of 30% of casein and 10% of cottonseed oil (Wesson Oil Co., New Orleans, La.); all other diets contained 0.35% of iodinated casein.
[2] Body weight at three weeks; corresponding weights for first and third diets were 180 gm and 155 gm; rats in all other groups survived until they were killed at 4 weeks.

weeks; two groups which were unprotected and would not have survived for 4 weeks were killed at three weeks. Comparison of the first two groups shows the usual growth inhibition produced by feeding weanling rats the basal diet containing 0.35% of iodinated casein. In addition, the MR and the heart size were approximately doubled, whereas the cytochrome c concentrations in heart and liver were increased appreciably. The substitution of 10% of extracted LR in the diet for an equivalent amount of casein partially restored all of these hyperthyroid manifestations toward normal, but the effect of 15% of LR was much more impressive. When the amount of cottonseed oil in the 10% LR diet was 5, 10 or 15% (not included in table 1), the 4-week body weights were 170, 195 and 200 gm, respectively, whereas the corresponding MR's were 14.0, 12.7 and 11.8. The addition of 2% of cod liver oil to the 30% casein diet in the absence of LR had relatively little effect on the hyperthyroid state. Similarly the addition of 2% of cod liver oil to a diet containing 10% of LR gave slightly better growth than the LR alone, and decreased the MR by only 0.4 unit. A further addition of 10% of dry egg yolk to the diet containing 15% of LR and 2% of cod liver oil almost restored growth completely to the basal nonthyroid level. The other criteria of hyperthyrodism were also decreased slightly but not completely to the basal level. The addition of 10% of egg yolk to a diet containing 10% of LR plus 2% of cod liver oil increased the body weight at 4 weeks from 205 to 225 gm and decreased the MR from 12.1 to 9.5.

These results indicated that the LR protected weanling rats from the toxic effects of thyroid feeding by counteracting the hyperthyroid state itself. Dietary oils in excess of the 10% of cottonseed oil normally present had some, but relatively little, beneficial effect on the MR or growth rate. Egg yolk appeared to act like LR in decreasing the MR when the 10% of dietary LR was insufficient for a maximal response.

Metabolic rate (MR) response with time. The relative rates at which the MR increased with different amounts of dietary iodinated casein are shown in figure 1. The MR of the group fed the basal diet remained constant at 8.7 for 7 weeks. With 0.05 to 0.50% of iodinated casein in the diet, all groups reached a maximal plateau in the MR

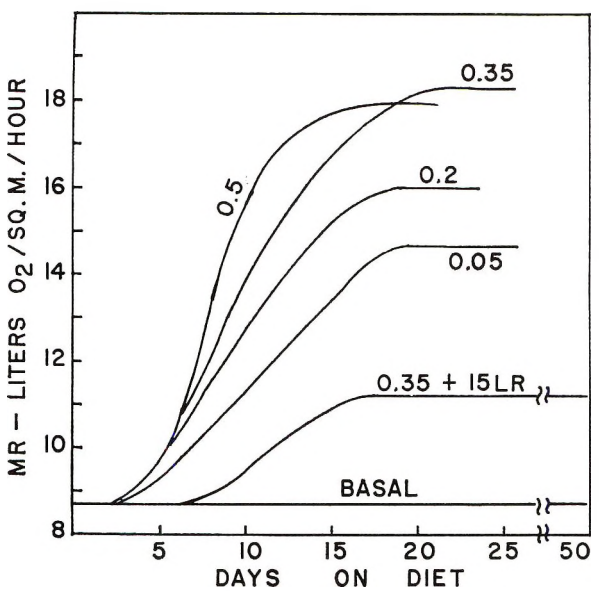

Fig. 1 Effect of feeding 0.05, 0.2, 0.35 and 0.5% of iodinated as well as 0.35% of iodinated casein plus 15% liver residue in the diet of weanling rats on the metabolic rate (MR).

after about 20 days and remained at that point until death from thyrotoxicosis occurred (usually between the third and fourth week). With 15% of LR in the diet containing 0.35% of iodinated casein, the maximal MR was achieved in less than three weeks and remained constant thereafter for at least 7 weeks. These results demonstrated that a constant steady-state MR could be determined after about three weeks of feeding the various diets.

Iodinated casein compared with liver residue concentration. Figure 2 shows the steady-state MR achieved with various dietary combinations of iodinated casein and LR. A relative titration of the iodinated casein by the LR is evident. The MR effects of 0.05% of dietary iodinated casein were almost completely eliminated by including 10% of LR in the diet. With larger amounts of dietary iodinated casein this protection by LR was progressively less, but in proportion to the relative amounts of each factor present.

When the relative amounts of LR and iodinated casein were such that the steady-state MR exceeded approximately 14, the rats succumbed rapidly to the thyrotoxicosis, and one-half or more of the rats in each group were dead by 4 weeks and nearly all by 5 weeks. With a steady-state MR of around 12, most of the rats survived for 4 weeks, but some died sporadically thereafter. When the combination of iodinated casein and LR maintained the MR below 12, nearly all of the rats survived for at least 6 weeks. Between the sixth and tenth week of this experiment, probably as a result of the spring seasonal change, the MR of the basal control group spontaneously declined 2.0 units (from 8.7 to 6.7), and the MR of the groups receiving various combinations of iodinated casein and LR which kept the MR below 12 also decreased by about the same. In comparison with the controls the steady-state MR of the groups receiving iodinated casein plus LR remained relatively constant from the third to the tenth week. Thus, an increased MR of about 40% could be tolerated by growing rats for 6 to 10 weeks at the environmental temperature prevailing during this experiment, whereas an increase of approximately 60% caused rapid fatality. The same MR was produced by different combinations of LR and iodinated casein, and all such combinations had the same effect on survival.

Liver residue did not counteract completely the effect of iodinated casein on

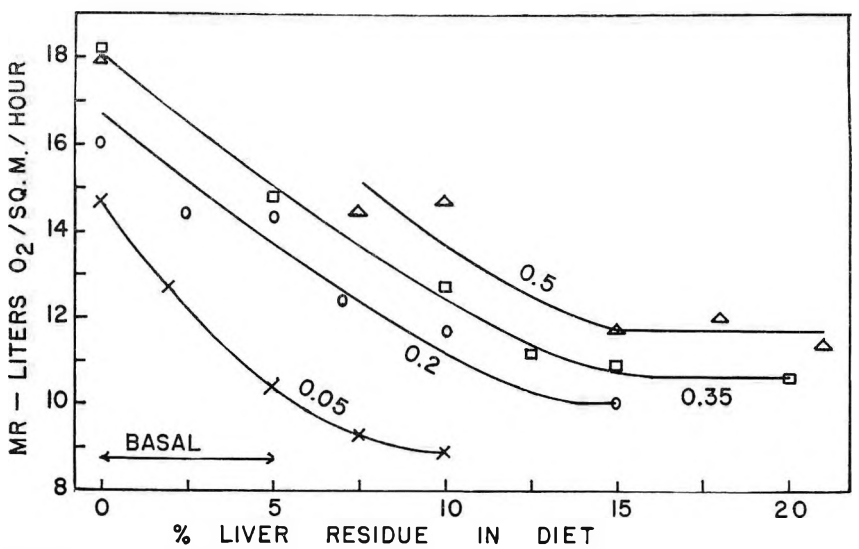

Fig. 2 Metabolic rate (MR) response in relation to the relative amounts of iodinated casein and liver residue in the diet. Weanling rats were fed diets containing 0.05 to 0.5% of iodinated casein plus zero to 20% of extracted liver residue, and the steady-state MR was determined after three weeks.

the MR irrespective of the amount fed. Fifteen per cent of LR gave a maximal reduction in MR with either 0.35 or 0.5% of dietary iodinated casein, and no further reduction occurred when this was increased to 20% of LR. The magnitude of this "residual MR" varied with the amount of iodinated casein in the diet and amounted to approximately one MR unit above the basal level when the diet contained 0.1% of iodinated casein (plus 10, 15 or 20% LR) and three units for 0.5% of iodinated casein.

Rats fed 1.0% of iodinated casein in a basal 35% casein diet all died between the second and third week of the experiment with an MR of 14.9 ± 0.38 (SE). When 17% of LR was substituted for an equivalent amount of casein, the MR was 10.3 ± 0.36 and all rats survived for at least 4 weeks. When 10% of egg yolk was added in addition to the LR, the MR was reduced further to 8.4 ± 0.41. In the absence of iodinated casein, an 18% casein plus 17% LR diet gave an MR of 7.1 ± 0.40, whereas rats fed the basal 35% casein diet had an MR of 6.3 ± 0.23. Hence, LR per se may have increased the MR slightly, but certainly did not counteract the rat's endogenous thyroidal activity. The lack of any inhibition of the endogenous MR by dietary LR was confirmed by additional studies in which rats fed a basal 30% casein diet had an MR of 7.1 ± 0.33, whereas those fed 15 to 20% of extracted or unextracted liver residue all had MR's varying from 7.3 to 7.9 (SE = 0.24 to 0.32), and rats fed zero casein plus 37.5% of unextracted LR had an MR of 8.4 ± 0.34.

Growth. Figure 3A shows the growth response of weanling rats to various combinations of dietary iodinated casein and liver residue. With 0.05% of iodinated casein a maximal effect on growth was achieved with 5% of LR, and this restored the growth rate almost to the basal nonthyroid level. With more iodinated casein in the diet, an optimal effect on growth was achieved with 7 to 10% of LR; larger amounts of LR reduced the MR still further, but did not promote further growth. The 4-week growth increment produced by an optimal concentration of LR increased from 45 to 60 gm as the iodinated casein content decreased from 0.5 to 0.05%.

Figure 3B shows the growth response of thyrotoxic rats in relation to the MR

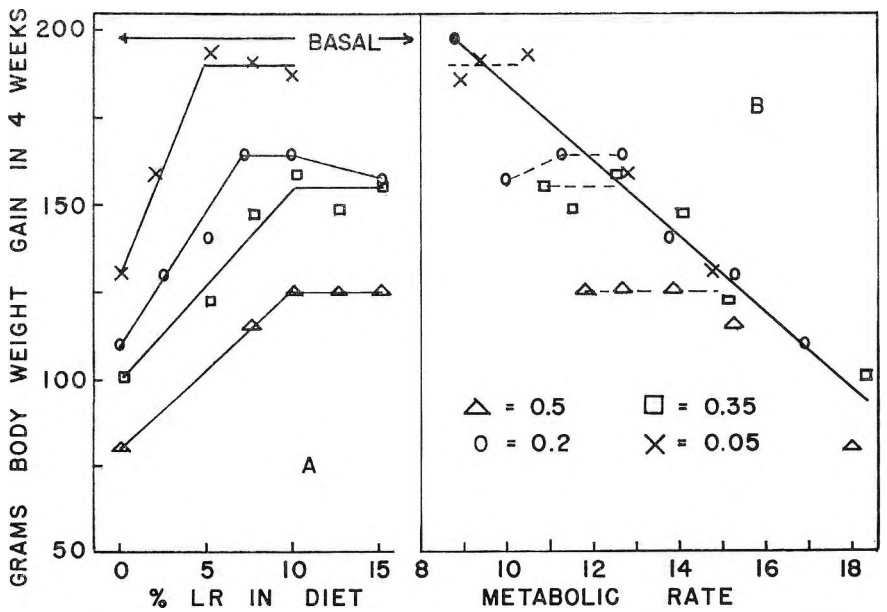

Fig. 3 Growth of thyrotoxic rats in relation to (A) the relative amounts of iodinated casein (as indicated by the symbols) and liver residue in the diet, and (B) the resulting metabolic rate in liters of O_2 consumed per square meter of body surface per hour.

achieved with various combinations of iodinated casein and LR. With 0.05, 0.2 or 0.35% of iodinated casein in the diet, all the experimental values fell along an inverse straight line which related MR to growth rate, except that deviations from this line occurred when the LR exerted no further effect on growth but was still effective in further decreasing the MR. Except for this limitation, the growth response to LR could be attributed directly to its effect in reducing the MR, and this exception simply showed that some other limitation on growth occurred before the maximal reduction in MR was achieved. The values obtained with 0.5% of dietary iodinated casein deviated from this line somewhat, but it was not clear whether this deviation was real or artifactually due to difficulties in obtaining consistent MR values at this high level of iodinated casein.

The liver residue used in these studies inhibited growth somewhat when fed at concentrations higher than 10% of the diet. For example, rats fed a thyroid-free basal diet of 14% of casein plus 20% of unextracted LR or 10% of casein plus 20% of extracted LR (alcohol plus $CHCl_3$ extracted) weighed 190 gm at 4 weeks, whereas those fed 30% of casein or 22% of casein plus 10% of unextracted LR weighed 220 gm. The addition of 10, 15 or 20% LR to a 30% casein diet containing 0.1% of iodinated casein all gave approximately the same growth response, and this was the same as that obtained by substituting the LR for an equivalent amount of casein (200 gm at 4 weeks).

Thyroxine and analogues. Liver residue counteracted the effect of dietary thyroxine (T_4) in the same way that it counteracted the effect of iodinated casein (table 2). Hence, the liver residue did not interfere with the digestion of the iodinated casein and from a quantitative standpoint had about the same activity with T_4 as with iodinated casein. Liver residue counteracted triiodothyronine (T_3) much less effectively than T_4 or iodinated casein. When T_3 was fed at 2 mg/kg of diet the substitution of 10 or 15% extracted LR in the diet for an equal weight of casein had no effect whatsoever on MR or survival. At lower concentrations of T_3, the LR did inhibit the increased MR, and was effective in promoting growth and survival. However, the range of T_3 concentration at which an effect of LR could be demonstrated was very narrow (0.6 to 1.0 mg/kg) by comparison with iodinated casein, and no concentration of iodinated casein was tested (0.05 to 1.0%) that was not counteracted to some extent by the LR. The metabolic effects of triiodothyroacetic acid, tetraiodothyroacetic acid and triiodothyropropionic acid were also counteracted effectively by LR. The response with diiodothyroacetic acid was similar, but LR was less effective in counteracting this analogue — possibly because of the large amount required to produce a metabolic effect.

Counteracting excess tissue thyroid. To determine whether dietary LR would hasten the removal or inactivation of excess thyroid hormone already in the tissues, weanling rats were fed the basal 30% casein diet containing 0.1% of iodinated casein for 15 days to build up the concentration of thyroid hormone in the tissues. One group continued to be fed the same diet as a control, while a second group was then fed the basal diet without iodinated casein and each of two other groups was transferred to a nonthyroid diet containing 30% casein plus 10% extracted LR, or 0.2% cholic acid (Westerfeld et al., '62). Starting on the fourteenth day and every two or three days thereafter, MRs were determined on all groups.

The results are shown in figure 4. Those rats which continued to receive iodinated casein after the fifteenth day reached the MR plateau of 13 about the twenty-first day. Rats receiving the basal diet only after the fifteenth day continued to have a high MR until the nineteenth day and it then decreased rapidly and reached the basal level of 7 about the twenty-fourth day. (The basal level was established by an additional group of rats fed the basal diet only throughout the experiment.) Rats receiving dietary LR or cholic acid after the iodinated casein was removed from the diet had an immediate decrease in MR which reached the basal level in 21 to 22 days. On the nineteenth day the differences in MR between the rats fed the basal diet only and those fed LR or cholic acid were significant at $P = 0.02$ for the single determinations carried out

TABLE 2

Effect of liver residue on the hyperthyroid activity of thyroxine and its analogues

Compound tested[1]	Diet conc.	Basal diet				Liver residue diet[1]			
		Metabolic rate[2]	Body wt 3-week	Body wt 4-week	Survival 4-week	Metabolic rate[2]	Body wt 3-week	Body wt 4-week	Survival 4-week
	mg/kg								
Basal	—	6.2 ± 0.24	178	226	5/5	—	—	—	—
Iodinated casein	1,000	16.5 ± 0.42	151	168	2/5	8.7 ± 0.26	175	225	5/5
T_4	10	15.4 ± 0.51	151	171	2/6	8.1 ± 0.31	169	205	5/5
Basal	—	6.2 ± 0.21	204	253	5/5	—	—	—	—
T_3	2	14.2 ± 0.46	138	159	2/6	14.7 ± 0.73	155	187	3/6
	1	12.5 ± 0.49	144	153	2/6	9.6 ± 0.34	183	212	5/5
	0.8	13.1 ± 0.41	148	—	1/6	8.1 ± 0.48	199	240	4/5
	0.6	11.4 ± 0.36	175	195	4/6	7.9 ± 0.36	188	233	5/5
	0.4	8.7 ± 0.30	190	222	6/6	8.4 ± 0.26	188	233	5/5
Basal	—	5.5 ± 0.19	199	241	8/8	—	—	—	—
Diac	300	9.7 ± 0.25	155	173	8/9	8.7 ± 0.30	175	197	9/9
Triac	3	10.2 ± 0.52	155	170	7/9	7.4 ± 0.27	188	219	9/9
Tetrac	13	10.7 ± 0.41	153	174	6/9	7.6 ± 0.27	185	212	9/9
Triprop	6	9.5 ± 0.28	163	191	8/9	7.4 ± 0.32	183	218	8/9

[1] The following compounds were added to the basal diet of 30% casein or to a liver residue diet containing 20% of casein plus 10% of extracted liver residue (for the iodinated casein and T_4 experiments) or to 20% of casein plus 15% of unextracted liver residue (for the other tests).

T_4 = L-thryroxine, pentahydrate, Na salt; T_3 = L-triiodothyronine, Na salt (Smith, Kline & French, Philadelphia); Diac = diiodothyroacetic acid; Triac = triiodothyroacetic acid diethanolamine salt; Tetrac = tetraiodothyroacetic acid, Na salt (Aldrich Chemical Company, Milwaukee); Triprop = triodothyropropionic acid (Warner Lambert, Morris Plains, N. J.).

[2] MR in liters O_2/m^2 of body surface/hour ± standard error of the mean. The effect of liver residue was significant at $P = 0.01$ for all tests except Diac ($P = 0.02$) and the 2 and 0.4 mg/kg concentrations of T_3.

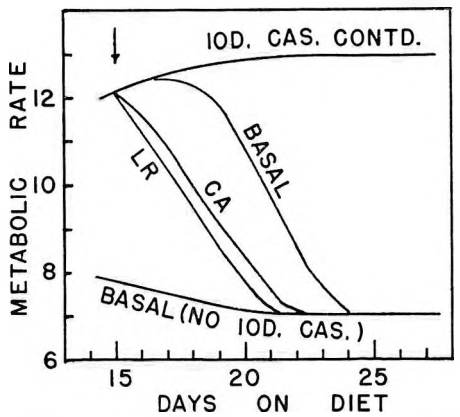

Fig. 4 Effect of subsequently feeding 10% of liver residue (LR) or 0.2% of cholic acid (CA) in the diet, on the elevated metabolic rate (liters O_2/m^2/hour) produced by prefeeding 0.1% of iodinated casein for 15 days.

on each rat in this experiment. Rats fed these protective substances all gained 90 gm (from 140 gm to 230) in body weight during the two weeks they were fed those diets, whereas the rats fed the basal diet only, after the iodinated casein gained 70 gm. Thus the inclusion of LR or cholic acid in the diet either hastened the removal of, or in some other way helped to counteract, the biochemical effect of excess thyroid hormone already in the tissues. A possible alternate explanation, i.e., that these substances counteracted some residual intestinal iodinated casein, appears unlikely on the basis of the time involved. No effect on MR was observed for 4 days when 0.1% of iodinated casein was first fed, and in this experiment it required 4 days after withdrawal of the iodinated casein before the MR began to decrease. When the diet contained LR, the decrease in MR was quite marked by 4 days.

Parenteral thyroxine and liver residue (LR). Dietary LR was less effective in counteracting the effects of T_4 when the latter was injected subcutaneously as compared with oral administration. Table 3 shows the results of two such experiments; the reduction in MR produced by feeding LR was significant at $P = 0.01$ in some but not all experiments. Growth and survival data also demonstrated the protection by LR and cholic acid. In the absence of T_4 administration neither the cholic acid nor the LR decreased the MR (7.8 ± 0.29 and 7.5 ± 0.33, respectively).

DISCUSSION

Previous reports have indicated that liver residue did not affect the increased metabolic rate produced by thyroid feeding. Ershoff ('47) reported no difference in the basal metabolic rate of rats fed 0.5% of desiccated thyroid when either 10% of liver or yeast was included in the diet, but no studies were made to determine the actual effect of the liver on the MR by comparison with an unprotected hyperthyroid group. Similarly Ershoff ('49) found an increase in the MR of rats fed a soybean meal diet containing 0.25 to 0.5% of desiccated thyroid or 0.125% of iodinat-

TABLE 3

Effect of thyroxine (T_4) administered subcutaneously to rats being fed the basal diet with or without liver residue or cholic acid[1,2]

Exp. no.	Diet fed	T_4	Metabolic rate[3]	Body wt	
				3-week	4-week
				gm	*gm*
1	Basal	—	7.4 ± 0.26	180	225
	Basal	+	12.9 ± 0.55	150	175[4]
	Liver residue, 15%	+	11.5 ± 0.34	150	190
	Cholic acid, 0.2%	+	11.7 ± 0.42	165	210
2	Basal	—	7.3 ± 0.30	244	—
	Basal	+	15.1 ± 0.50	184	—
	Liver residue, 15%	+	11.6 ± 0.61	211	—

[1] Exp. 1: 50 μg of T_4 dissolved in 0.1 N NaOH, diluted with saline to 0.2 ml, and injected twice daily into weanling male rats (50 gm) for 4 weeks.
[2] Exp. 2: 200 μg of T_4 injected every other day for 3 weeks into rats weighing 104 gm initially.
[3] Metabolic rate in liters of O_2 consumed/m^2 of body surface/ hour, as determined between the third and fourth week.
[4] Forty per cent (2/5) survival; 100% survival in all other groups.

ed casein, but did not determine whether the soy diet gave a lower MR than would have been obtained with the same amount of thyroid substance added to a casein diet; nor did Stevens and Henderson ('58) observe an effect of 10% of liver residue on the MR of rats fed 0.243% iodinated casein (with a 3.07% thyroxine equivalence), but it seems probable that these results were negative because of the very large amount of thyroidal activity used. None of the previous studies therefore are directly contradictory to the results being reported, although they also would not have suggested or predicted them. In retrospect, an antithyroidal effect might have been anticipated from the way liver residue counteracted the adrenal and ventricular hypertrophy, the atrophy of the thymus and lack of ovarian development seen in hyperthyroid rats, for none of these responses would be anticipated from a growth factor that did not influence the hyperthyroidism itself.

The mechanism by which liver residue counteracts the metabolic effects of the thyroid hormone is not clear. It might have (1) interfered with the intestinal absorption of the thyroactive material; (2) hastened the removal of the thyroid hormone from the tissues; or (3) inhibited the peripheral utilization or functioning of the thyroid hormone. The counteracting of parenteral thyroxine suggests a peripheral site of antagonism, but such results might also be the consequence of a more rapid excretion or destruction of the hormone, and this latter possibility gains some credence from the activity of the bile acids. The very rapid effect of liver residue in restoring the hyperthyroid metabolic rate to normal also suggests a peripheral antagonism or a rapid removal of peripheral hormone. The lack of any decrease in the endogenous metabolic rate suggests that, irrespective of mechanism, the liver residue is primarily concerned with counteracting the excess hormone that was administered rather than the thyroid hormone normally present. Additional studies will be required to clarify the mechanism by which liver residue counteracts the metabolic effect of the thyroid hormone.

SUMMARY

1. Liver residue counteracted the metabolic effects of iodinated casein in proportion to the relative amounts of the two substances in the diet, but did not restore the metabolic rate completely nor decrease the endogenous metabolic rate.

2. Growth and survival simply reflected the magnitude of the effect of liver residue in reducing the metabolic rate; with large amounts of liver residue some other limitations on growth occured before the maximal reduction in metabolic rate was achieved.

3. Liver residue counteracted the metabolic effects of dietary thyroxine, triiodothyronine, triiodothyroacetic acid, tetraiodothyroacetic acid and triiodothyropropionic acid, but was less effective against diiodothyroacetic acid. It also counteracted subcutaneous thyroxine, but less effectively than dietary thyroxine. The range of concentration of triiodothyronine within which an effect of liver residue could be demonstrated was very narrow.

4. The high metabolic rate produced by prefeeding iodinated casein was restored to normal more rapidly when the diet contained liver residue or cholic acid.

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An Antithyrotoxic Assay Based Upon the Metabolic Rate Response[1,2]

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All previous nutritional studies related to the antithyrotoxic factor have utilized growth and survival of thyroid-fed rats as the principal criteria of antithyrotoxic activity. With the recognition (Westerfeld et al., '62) that liver residue exerted its antithyrotoxic effect on growth and survival as the secondary consequence of a reduction in the metabolic rate, it was possible to develop an assay procedure for antithyrotoxic activity which was based upon the metabolic rate response. Such an assay adds an additional parameter to the other criteria of antithyrotoxic activity without interfering with the usual measurements of growth and survival. It further provides a means of determining whether all substances that exhibit antithyrotoxic activity on the basis of growth and survival also have an effect on the metabolic rate.

METHODS

From the previous studies (Westerfeld et al., '62) a low concentration of iodinated casein[3] in the diet appeared to be advantageous for the development of a sensitive assay for antithyrotoxic activity by the metabolic rate response. A concentration of 0.1% of iodinated casein (1% thyroxine equivalent) was therefore incorporated into the basal 30% casein plus 10% cottonseed oil[4] diet previously described (Westerfeld et al., '62), and fed to groups of 5 weanling male rats of the Holtzman strain (approximately 50 gm body weight) for 4 weeks. Test substances were substituted for sucrose in the diet, except that when various proteins were added at concentrations of 20%, the casein was reduced to 20%. Growth and survival were recorded weekly, and metabolic rates were determined three times on each rat between the nineteenth and thirtieth days as previously described (Westerfeld et al., '62). The unprotected hyperthyroid controls and those rats that exhibited minimal protection on the basis of growth curves were analyzed first in order to obtain the metabolic rate data before the rats succumbed to the thyrotoxicosis.

RESULTS

Figure 1 shows the metabolic-rate, dose-response curve obtained when weanling male rats were fed increasing amounts of liver residue (LR) together with 0.1% of dietary iodinated casein. The 10% liver residue diet has arbitrarily been equated with 100 units of LR activity. Essentially the same curve was obtained when the LR was substituted for casein as when the LR was added to a constant 30%

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[2] Materials used in this study were obtained as follows: Protamone and Cerophyl: Cerophyl Laboratories, Kansas Ctiy, Missouri; triiodothyronine: Warner-Lambert, Morris Plains, New Jersey; casein (vitamin free), egg yolk, brewer's yeast, lactalbumin, fibrin, egg albumin, hemoglobin, methionine and AET: Nutritional Biochemicals Corporation, Cleveland; liver residue: Wilson and Company, Chicago; fermentation residue (Omafac): E. R. Squibb and Sons; fish solubles: Philip R. Park, Incorporated, San Pedro, California; distiller's solubles: Schenley Distillers, Louisville; whey: Western Condensing Company, Appleton, Wisconsin; soy protein (ADM C-1, Assay Protein): Archer-Daniels-Midland Company, Minneapolis; cottonseed meal: Eufaula Cotton Oil Company, Eufaula, Alabama; wheat gluten: General Biochemicals Inc., Chagrin Falls, Ohio; fish meal: Viobin, Monticello, Illinois; gelatin: Baker and Adamson, New York; bile acids: Steraloids, Queens, New York; Guanethidine: Ciba, Summit, New Jersey; thiouracils: Mann Laboratories, New York; dibenzyline: Smith, Kline and French, Philadelphia; reserpine: California Biochemicals, Los Angeles; dihydroergotamine: Sandoz, Hanover, New Jersey; antibiotics: Bristol Laboratories, Syracuse, New York.
[3] Protamone, Cerophyl Laboratories, Kansas City, Missouri.
[4] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

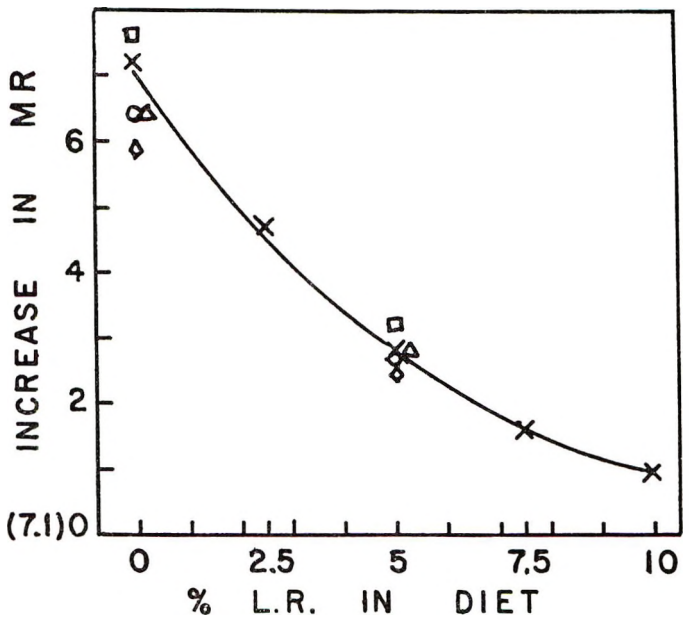

Fig. 1 An assay curve for liver residue which shows the increased metabolic rate (above the average basal of 7.1 liters of O_2 consumed per square meter body surface per hour) resulting from the inclusion of zero to 10% of extracted liver residue in a diet containing 0.1% of iodinated casein. The individual symbols illustrate the reproducibility of the curve in 5 independent experiments.

casein. The individual points plotted for zero and 5% LR in figure 1 illustrate the variability obtained in reproducing the curve in the first 5 independent experiments.

This assay response has been used satisfactorily in our laboratory for over a year. We have routinely included with each run: (1) a nonthyroid basal control; (2) an unprotected hyperthyroid group receiving 0.1% of iodinated casein; (3) a standard 5% liver residue plus 0.1% of iodinated casein group; and (4) sometimes a similar 10% liver residue standard. These controls provided an independent reference curve for each experiment which was generally in good agreement with the assay curve shown in figure 1. The first 11 independent assay runs gave an average nonthyroid basal rate of 7.17 (range, 7.0 to 7.3; standard deviation, ± 0.185); the unprotected 0.1% iodinated casein group averaged 13.53 (11.8 to 15.1; SD, 1.06), and the 5% liver residue standard averaged 10.24 (8.8 to 11.3; SD, 0.79). In the next 5 runs the basal nonthyroid metabolic rate averaged 6.16 ± 0.27 (SD) and both the unprotected hyperthyroid and the 5% LR standard groups also decreased by about one unit (13.14 ± 1.41; 9.28 ± 0.87). The reason for the decreased metabolic rate in the basal control group is unknown (possibly seasonal or environmental temperature), but the relative differences in metabolic rates remained unchanged, and the same assay curve was applicable to these differences. The assay was not very precise in either the very low (< 20 units) or very high (60 to 100 units) ranges — the former because of the variability in reproducing the same exact metabolic rate with the unprotected hyperthyroid rats — the latter because of the "flattening" of the assay curve at the higher concentrations of LR. The assay could undoubtedly be made more precise by the use of larger groups of rats, but with the procedure described there was no difficulty in distinguishing between good, moderate, and poor sources of antithyrotoxic activity.

Distribution of antithyroid activity.[5] Table 1 summarizes the approximate ac-

[5] See footnote 2.

TABLE 1

Approximate antithyrotoxic activity of various substances as determined by the metabolic rate response assay procedure

Diet conc.	Substance tested	
%		*units*[1]
1	Liver residue conc. (Overby)	85
10	Fermentation residue	70
10	Fish meal	60
10	Cottonseed meal	55
10	Distiller's solubles	50
10	Rabbit muscle residue	45
10	Egg yolk	25
10	Thymus (dessicated)	25
40	Yellow corn meal	25
40	Extracted soy flour[2]	25
10	Dried grass[3]	15
10	Brewer's yeast	10
10	Whey	10
10	Wheat germ	10
5	Hemoglobin	90
0.2	Cholic acid	35
0.2	Na glycocholate	55
0.2	Deoxycholic acid	45
0.2	Dehydrocholic acid	60
23	Cottonseed oil, refined[4] (total)	28
23	Cottonseed oil ± 0.115% iodinated casein	25
5	Liver residue fat	70
2	Cod liver oil	12
5	Olive oil	28
20	Lactalbumin[2]	75
20	Soy protein[2]	35
20	Fibrin[2]	55
mg/kg		
15	Guanethidine	30
500	6-Propyl thiouracil	45
100	Dibenzyline	20

[1] The standard 10% liver residue diet was arbitrarily equated with 100 units of LR antithyrotoxic activity, and all other metabolic rate responses were converted to units by means of the assay curve in figure 1.
[2] Added to a 20% casein diet.
[3] Cerophyl, Cerophyl Laboratories, Kansas City, Missouri.
[4] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

tivity of various test substances, as determined by the metabolic rate response. Of the natural products tested, fermentation residue, fish meal, cottonseed meal, distiller's solubles and a rabbit muscle residue (prepared like liver residue) were 45 to 70% as active as the standard liver residue. Egg yolk and thymus were only about one-fourth as active as LR, and corn meal, dried grass,[6] brewer's yeast, fish solubles, whey and wheat germ were relatively inactive. A liver residue concentrate prepared by Dr. L. R. Overby was 8.5 times as active as the original liver residue.

Cholic acid reduced the metabolic rate of the thyroid fed rats; deoxycholic, dehydrocholic, and glycocholic acids were even more active than the cholic acid. Lithocholic acid (0.2%) and hyodeoxycholic acid (0.1%) were essentially inactive in the MR test, and they also had no effect on growth or survival. Cholesterol (1%) had little or no effect on the MR or growth, and also had no synergistic effect when combined with 0.2% of cholic acid.

An increase in the dietary cottonseed oil from the usual 10% to a total of 23% had a relatively small but definite effect on the MR. This could theoretically have been due to the consumption of less iodinated casein in the higher-caloric diet, but when this was compensated by increasing the iodinated casein from the usual 0.1 to 0.115%, the antithyrotoxic effect of the oil was still observed. All fats were not equally effective in this respect, and this response therefore cannot be attributed to a general increase in the caloric content of the diet. The relative activities of the fats tested were in the order: liver residue fat, 70; cod liver oil and olive oil, 30; cottonseed oil, 10; corn oil and lard were inactive. The liver residue fat was the combined material extracted from liver residue by hot 95% alcohol and then chloroform.

Both cottonseed meal and its oil were active in the same way that both liver residue and its extractable fat were active. In both cases two-thirds or more of the total activity remained with the lipid insoluble fraction. Soybean oil and its meal were also active in growth and survival experiments (Ershoff, '49). Either a small amount of the free antithyrotoxic factor can be extracted into the lipid fraction, or a small portion of the total activity is present in a lipid-soluble form. The latter possibility is suggested by the fact that when a petroleum ether solution of the liver residue fat was extracted exhaustively with $Ba(OH)_2$, all of the activity remained in the solvent. The free liver residue factor is not soluble in petroleum ether, and if carried into solution by the large amount of lipid present, it should have been removed by the $Ba(OH)_2$. It is suggested that the bulk of the antithyro-

[6] Cerophyl.

toxic activity is present in liver in a lipid-insoluble form, but that a small portion is also present as a lipid.

The addition of 20% of lactalbumin or fibrin to a diet containing 20% of casein gave an impressive decrease in the MR, whereas 20% of soy protein gave a modest response. Increasing the casein content from the usual 30% to a total of 40% had only a small effect. The addition of 10% of egg albumin or gelatin to a 30% casein diet had no effect on the MR, and these two proteins were also inactive when 20% concentrations were added to a 20% casein diet. Wheat gluten was moderately active in this test, and hemoglobin[7] was the most active naturally occurring material yet tested (other than the bile acids). A possible beneficial effect from a high protein diet in thyrotoxicosis can be postulated in general terms, but it is much much more difficult to see how additional protein would reduce the MR, or why different proteins would behave differently, unless some factor other than amino acids was associated with the active proteins.

The effectiveness of soy protein in counteracting the hyperthyroid effects of iodinated casein was unexpected in view of our previous results with soy flour diets (Westerfeld and Richert, '52). In those studies a 40% soy protein diet (supplied as 80% of extracted soy flour containing 50% of protein) was less effective in protecting weanling rats fed 0.1% of iodinated casein than was a purified 31% casein diet. However, additional experiments demonstrated the following. In the absence of iodinated casein, rats fed a 40% soy protein diet (with or without the addition of 0.75% DL-methionine) grew at the same rate (255 gm at 4 weeks) and had the same MR (6.8 to 7.4) as rats fed a 30% casein diet (7.3). In the presence of 0.1% of iodinated casein, all rats fed the 40% soy protein diets (± methionine) survived for 4 weeks, weighed approximately 215 gm, and had a MR of 9.3; all rats fed the 30% casein diet died between the third and fourth week with a MR of 15.1 and an extrapolated 4-week body weight of 175 gm. A further addition of 5% of LR to the soy protein diets allowed nearly normal growth (250 gm) with a MR of 8.3 to 8.6, whereas a similar addition of 5% of LR to the casein diet reduced the MR to 10.4 and gave a 4-week body weight of 205 gm with 80% survival. Rats fed 80% of extracted soy flour plus 0.5% of methionine as the sole source of protein rejected and scattered the diet, weighed only 165 gm at 4 weeks, and had an MR of 10.5.

Guanethidine possessed measurable activity by the MR test, and 6-propyl thiouracil was reasonably active by this criterion. The latter had no effect on growth. Dibenzyline may have had slight activity.

The following substances were completely inactive by the metabolic rate as well as the growth criterion at the concentrations tested (mg/kg of diet): reserpine (1.5), vitamin K_1 (330), vitamin C (500), Ca pantothenate (3,000), $NaHSO_3$ (2,000) 2-aminoethylisothiouronium bromide (AET) (2,000), dihydroergotamine (10), tyrosine (5,000), and polyoxyethylene sorbitan trioleate (Tween 85) (10,000).

In two experiments procaine penicillin (30 mg/kg of diet) had good antithyrotoxic activity by the metabolic rate criterion as well as by growth and survival. However, in numerous additional studies it was inactive by all criteria. In two of the latter studies, the following antibiotics were also inactive: 20 mg/kg of penicillin G or tetracycline; 100 mg/kg of kanamycin, streptomycin or staphcillin; 500 mg/kg of chloramphenicol; 1,000 mg/kg of sulfanilamide, sulfapyridine, or sulfasuxidine.

Growth and survival. The conditions used in this assay for the determination of antithyrotoxic activity by the metabolic rate effect provided less than the optimal concentration of iodinated casein for the study of growth and survival. Minimal protection allowed most of the rats to survive the 4-week experiment, and survival was therefore of little or no value in quantitating the response. Growth curves were very useful since the growth response paralleled the metabolic rate effect, but the growth response was somewhat erratic in repeated experiments at this low concen-

[7] The marked antithyrotoxic activity of commercially available hemoglobin was first observed by Dr. L. R. Overby, Abbott Laboratories, North Chicago, Illinois (personal communication) in growth and survival studies, and was found by him to be associated with the insoluble (protein) rather than the acid-acetone soluble (heme) portion of the preparation.

tration of iodinated casein. The average 4-week body weights for all the runs were: with nonthyroid basal, 230 gm; with 0.1% of iodinated casein, 170 gm; and with 0.1% of iodinated casein plus 5% LR, 200 gm.

No substance tested gave a good growth response without also reducing the metabolic rate. Liver residue, hemoglobin, fermentation residue, cottonseed meal, liver residue fat, lactalbumin and fibrin were all very active by both the MR and growth criteria. Only 6-propyl thiouracil had a sizeable effect on the MR without giving a corresponding growth response, and this could have been the result of a growth-retarding toxicity of the substance unrelated to the antithyroid response. All other substances gave reasonably parallel responses between growth and metabolic rate.

Females. In two experiments, female rats appeared to be less satisfactory than males for the study of antithyrotoxic activity. There was less inhibition of growth of the females by feeding iodinated casein, and there was a smaller reduction of the elevated MR as a result of including liver residue in the diet. The magnitude of both parameters of antithyrotoxic activity was therefore decreased, and the difficulty of quantitating the result was correspondingly increased. Nevertheless, it was possible to show that the females responded in a like manner to the males to the various dietary supplements. The following substances exhibited good antithyrotoxic activity in both males and females: liver residue and its concentrate, hemoglobin, liver residue fat, fermentation residue, cottonseed meal, lactalbumin, fibrin and deoxycholic acid. The following were essentially inactive in both males and females: whey, dried grass, wheat germ, corn oil, cholesterol, egg albumin. The following were at least moderately active in both sexes: soy protein, distiller's solubles and sodium glycocholate.

The inhibition of the normal development of the ovaries and uterus produced by iodinated casein was largely or completely overcome by all those substances that effectively reduced the MR. The basal nonthyroid uterine weight of approximately 160 mg/100 gm of body weight was reduced to about 50 by feeding iodinated casein and was restored to more than 120 by liver residue and its concentrate, liver residue fat, cottonseed meal, fermentation residue, hemoglobin, dehydrocholic acid, deoxycholic acid, sodium glycocholate, lactalbumin, soy protein, fibrin and distiller's solubles. Those substances that had little or no effect on the MR and did not allow any significant development of the ovaries or uterus included: dried grass, whey, procaine penicillin, and corn oil.

Antithyrotoxic studies with triiodothyronine

All of the nutritional studies related to the antithyrotoxic factor previously published utilized iodinated casein, desiccated thyroid, thyroglobulin, or thyroxine (T_4) as the thyroactive material. The metabolic effect of triiodothyronine (T_3) was also found to be counteracted by liver residue (Westerfeld et al., '62), but the effect was limited to a narrow range of T_3 concentration, and the magnitude of the inhibition appeared to be less than that produced with iodinated casein or thyroxine. This suggested that different substances might have different effects with respect to T_3 and T_4, and the following studies with T_3 were designed to test this possibility.

Weanling male rats were fed the basal 30% casein plus 10% cottonseed oil diet containing 0.6, 0.8, or 1 mg/kg of T_3. The MR increased rapidly (fig. 2) and stabilized at a maximal value after 16 to 18 days. The MR of rats protected against T_3 by the inclusion of cottonseed meal in the diet reached a maximal value in about 14 days. Hence, for assay purposes, the steady-state MR was determined after feeding the diet for 16 days. The MR response to T_3 was similar to that obtained with iodinated casein except that it increased more rapidly and correspondingly stabilized at the maximal value a few days earlier.

Figure 3 shows the average assay curves obtained when zero to 30% of cottonseed meal or zero to 15% of liver residue was tested in diets containing 0.8 or 1 mg/kg of T_3. The liver residue was twice as active as the cottonseed meal. The same results were obtained when the cottonseed meal

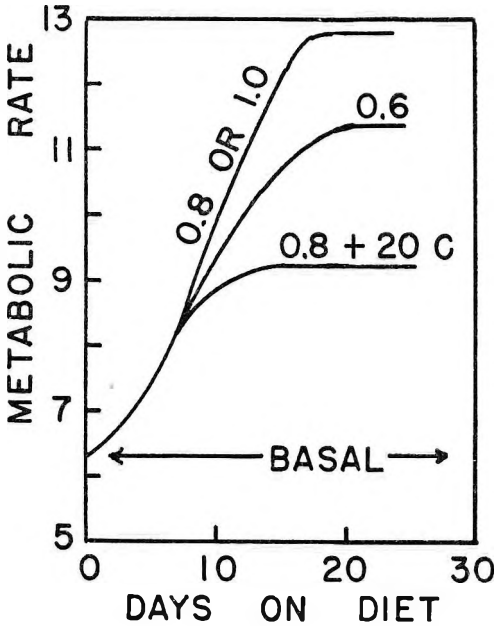

Fig. 2 Metabolic rate response (in liters of O_2 per square meter per hour) exhibited by weanling male rats fed diets containing 0.6, 0.8 or 1.0 mg of triiodothyronine/kg of diet. Metabolic rates are also shown for rats fed a nonthyroid basal diet as well as a diet containing 0.8 mg/kg of triiodothyronine plus 20% of cottonseed meal.

was added to a constant 30% casein diet as when it was substituted for a portion of the casein to keep the total protein constant at 30% (the cottonseed meal contained 40% protein). Neither 10, 20 or 30% of cottonseed meal had any significant effect on the endogenous MR in the absence of added T_3. Both curves represent the maximal sensitivity that can be achieved in this assay. A further reduction of T_3 concentration to 0.6 mg/kg gave a curve parallel to the two shown, but further displaced toward the base line and still covered the same relative response range. The characteristic features of this assay with T_3 are: (1) the high residual MR still remaining when a maximal reduction has been achieved with 15% of liver residue or 30% of cottonseed meal. and consequently, (2) a relatively small MR range (3 to 4 units) which corresponds to the significant portion of the assay curve. The residual MR with a diet containing 1 mg/kg of T_3 and 15% of liver residue was approximately 3 units, whereas a similar diet containing 0.1% of iodinated casein plus 15% of liver residue gave a residual MR of approximately 1 unit. The combination of 15% of LR plus 30% of cottonseed meal was no more effective against 1 mg/kg of T_3 than either one alone; hence, the residual MR could not be reduced further by larger amounts of the antithyrotoxic factor. The nature of these curves also demonstrated the reason that liver residue was relatively ineffective when the T_3 concentration was increased to as little as 2 mg/kg; the residual MR at that concentration would approach the unprotected level regardless of whether one-half of the T_3 might have been negated by the liver residue.

The three-week body weight of approximately 185 gm with the basal nonthyroid diet was reduced to approximately 145 gm when either 0.8 or 1 mg/kg of T_3 was fed. With the lower concentration of T_3, growth was completely restored to the nonthyroid level by 5% or more of liver residue or by 10% or more of cottonseed meal. With 1 mg/kg of T_3 in the diet, 10% of cottonseed meal gave a three-week body weight of 165 gm, whereas 20 or 30% of cottonseed meal restored growth completely. Growth was the same when the cottonseed meal was added to 30% casein or substituted for a portion of it.

Unprotected rats fed either 0.8 or 1 mg/kg of T_3 died a few days earlier than rats fed 0.1% of iodinated casein, and all such T_3 rats were usually dead by the 28th day. Survival data were not particularly informative except as an adjunct to the MR results; when the latter were decreased sufficiently, the rats survived; when the MR was high, the rats died sporadically.

Distribution of anti-T_3 activity. Different foodstuffs were assayed for their antithyrotoxic effects against T_3 by substituting them for an equal weight of sucrose in the 30% casein plus 10% cottonseed oil diet containing 0.8 mg/kg of T_3; when purified proteins were tested at 20% of the diet, the casein content was decreased to 20%. The diets were fed to groups of 5 weanling male rats, and the MR was determined in duplicate on each rat between day 16 and 28. The relative activity of the test substance was obtained from

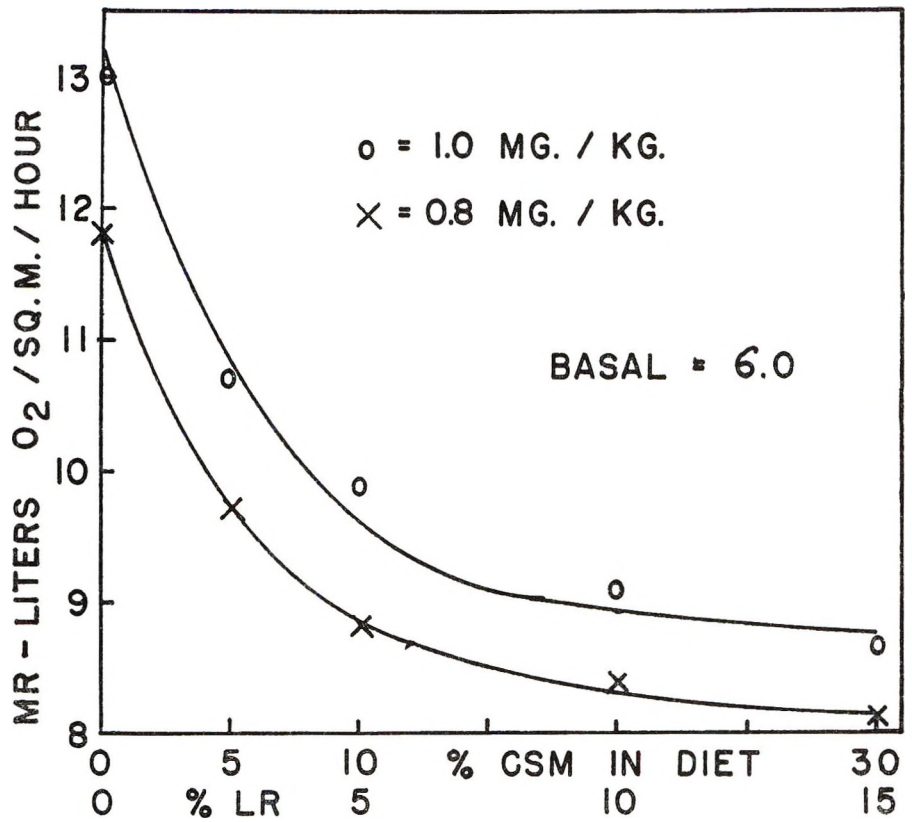

Fig. 3 The average assay curves for triiodothyronine (T_3) obtained by plotting the metabolic rate (MR) against the concentration of cottonseed meal (CSM) or liver residue (LR) in the diet. Weanling male rats were fed a basal 30% casein plus 10% cottonseed oil diet containing 0.8 or 1.0 mg/kg of T_3 and zero to 30% of cottonseed meal or zero to 15% of liver residue, and the metabolic rate was determined after 16 days. The curves for 1.0 mg/kg and 0.8 mg/kg of T_3 are the averages of 5 and 7 independent experiments, respectively.

an assay curve (fig. 3) which was run simultaneously and which included: (1) a basal nonthyroid group; (2) an unprotected group receiving 0.8 mg/kg of T_3; and (3) at least two cottonseed meal or liver residue standards. Because of the relatively small range of the MR response which is significantly in the assay curve, only semiquantitative evaluations were attempted. The results clearly distinguished between good, moderate and poor sources of anti-T_3 activity.

Those substances that had 60 or more units of antithyrotoxic activity against T_3 (as compared with 100 units for a 10% liver residue diet) included: 0.2% sodium glycocholate, 2% liver residue concentrate, 5% hemoglobin, 10% dry egg yolk, 20% fibrin and 20% lactalbumin. Those substances which were moderately active (30 to 50 units) included: 0.2% deoxycholic or dehydrocholic acid, 5% liver residue fat, 10% fermentation residue, brewer's yeast or cottonseed meal, and 20% soy protein. The following substances were relatively inactive (less than 15 units): 0.2% cholic or lithocholic acid, 10% whey, dried grass, wheat germ, thymus, corn oil, distiller's solubles, or dry fish solubles, 20% egg albumin or a total of 40% casein. Procaine penicillin (30 mg/kg of diet) was also inactive; 500 mg/kg of 6-propyl-thiouracil and 1,000 mg/kg of 2-thiouracil decreased the MR equivalent to 30 to 40 units of antithyrotoxic activity. In general, the growth response was inversely proportional to the effect of the diet on the MR. None of the

substances tested had a marked effect on growth without also decreasing the MR; hence they were fundamentally antithyroid rather than vitamin-like in their action.

In general, the same substances were active against both T_3 and iodinated casein, but the test with T_3 was more rigorous. For example, cholic acid had a moderate but easily measured effect against iodinated casein, but was essentially inactive against T_3. The other bile acids which had more activity than cholic acid against iodinated casein were still active against T_3. Similarly fermentation residue moved from the "good" category when tested against iodinated casein to the "moderate" category against T_3, and distiller's solubles moved from moderate to relatively inactive. Egg yolk was the only substance that appeared to be more effective against T_3 than against iodinated casein.

Adrenal weights. When either the 0.1% iodinated casein or 0.8 mg/kg of T_3 diets were fed to weanling male rats for 3 to 4 weeks, the degree of adrenal enlargement varied directly with the metabolic rate established by the diet. In different groups of rats fed the basal nonthyroid diet, the sum of both adrenal weights varied from 17 to 25 mg/100 gm of body weight; most of these groups had MR's close to 6. The adrenal weights increased from such starting values along a straight line whose slope varied somewhat in different experiments, but which usually was 3.5 to 4 mg/100 gm body weight for each unit increase in MR. Unprotected rats receiving either iodinated casein or T_3 had a MR which was approximately 6 units above the basal, and they had adrenal weights that were 20 to 25 mg/100 gm heavier than the controls. Those rats that were protected "maximally" by the inclusion of LR or cottonseed meal in the diet had adrenals heavier than those of the controls by an amount corresponding to the residual MR. When the adrenal weights were plotted against the corresponding MR in those experiments in which the antithyrotoxic activity of various foodstuffs was being tested, all of the values fell within experimental error along this straight line relationship. None of these substances had any effect on the adrenals which could not be associated with its effect on the MR, and the reversal of adrenal hypertrophy by the antithyrotoxic factor was probably another consequence of the lowering of the MR.

The increased MR which resulted from the feeding of 6 mg/kg of triiodothyropropionic acid, 3 mg/kg of triiodothyroacetic acid, 13 mg/kg of tetraiodothyroacetic acid, or 300 mg/kg of diiodothyroacetic acid (Westerfeld et al., '62) was accompanied by a corresponding increase in the adrenal weights, as described for iodinated casein and T_3. Similarly the inclusion of 15% of LR in these diets decreased the adrenal weights in accordance with its effect in lowering the MR.

The feeding of a diet containing 0.1% of iodinated casein or 0.8 mg/kg of T_3 to weanling male rats for 3 to 4 weeks did not produce any atrophy of the thymus, even though the MR was doubled.

DISCUSSION

In general the assay results obtained by the metabolic rate criterion agreed very well with those previously obtained by the criteria of growth and survival. The following substances have consistently been reported to have good antithyrotoxic activity: fermentation residues (Ershoff, '50; Overby and Fredrickson, '60a); distiller's solubles (Overby and Fredrickson, '60a; Dryden et al., '60); mammalian muscle (Overby and Fredrickson, '60b; Tappan et al., '53; Graham et al., '53; Boldt et al., '58); and kidney (Ershoff, '48, '50; Stewart and Henderson, '58). The following substances have been reported consistently to be relatively inactive: fish solubles (Overby and Fredrickson, '60a; Dryden et al., '60; Ershoff, '48); milk and milk products (Dryden et al., '60; Ershoff, '50; Tappan et al., '53); corn meal (Dryden et al., '60; Stevens and Henderson, '58; Tappan et al., '53); pancreas (Overby and Fredrickson, '60a; Stevens and Henderson, '58); and thymus (Overby and Fredrickson, '60a; Ershoff, '48). Variable responses have been reported for the following: fish meal and cottonseed meal were listed as good (Dryden et al., '60) or poor (Stevens and Henderson, '58); whole egg was reported to be good (Graham et al., '53; Overby et al., '59c) or poor (Tappan et al., '53); egg

yolk (Stevens and Henderson, '58) and egg albumin were poor (Graham et al., '53; Dryden et al., '60; Overby et al., '59c); dried grass was good (Dryden et al., '60), whereas alfalfa meal was poor (Overby and Fredrickson, '60a; Ershoff, '50, '59); brewer's yeast was good (Dryden et al., '60) or relatively inactive (Overby and Fredrickson, '60a; O'Dell et al., '55; Ershoff, '47, '48, '50; Stevens and Henderson, '58; Betheil et al., '47); spleen was good (Stevens and Henderson, '58) or poor (Overby and Fredrickson, '60a); wheat (Tappan et al., '53) and wheat germ were poor (Ershoff, '47) or moderate (O'Dell et al., '55), and gluten was moderately active (Graham et al., '53). A growth response has also been reported (Ershoff, '48) for heart, placenta, duodenum, brain, and lung (Stevens and Henderson, '58).

The antithyrotoxic effect of cholesterol has been variable in different laboratories and has been variable in different experiments within the same laboratory (Dryden et al., '60). Ershoff and Marx ('48) reported a minimal effect from cholesterol, inasmuch as it prolonged survival without affecting growth. Emerson et al.[8] and Page et al.[9] also reported that cholesterol was active, whereas Westerfeld and Richert ('52), Stevens and Henderson ('58) and Overby and Fredrickson ('61) obtained no antithyrotoxic response from cholesterol. The good protection afforded by a mixture of cholesterol plus bile salts (Marx et al., '48) can be attributed to the bile salts since the latter have consistently been found to be active (Overby et al., '59a).[10,11] In both the growth and survival tests (Overby and Fredrickson, '61) as well as by the metabolic rate response, deoxycholic acid was more effective than cholic acid, whereas lithocholic and hyodeoxycholic acids were inactive.

All investigators have described some degree of an antithyrotoxic response to an increased fat content of the diet (Ershoff, '49; Overby et al., '59a); this protective effect has generally been attributed to the presence of unsaturated fatty acids,[12,13] especially linoleic acid (Greenburg and Deuel, '50; Greenburg, '52). Cottonseed oil has been used extensively in these studies (Ershoff, '52, '53; Greenburg and Deuel, '50; Greenburg, '52), and a maximal response was reported for a total dietary concentration of 23% of cottonseed oil (Dryden et al., '60; Overby et al., '59b). All fats have not been found to be equally effective. Lard (Ershoff, '49; Westerfeld and Richert, '52) and numerous oils (Overby et al., '59b) afforded good protection when added to low fat diets, but the fat extracted from liver residue (Overby and Fredrickson, '60b) was appreciably more active than cottonseed oil, and the activity exhibited by olive oil was greater than could be accounted for by its linoleic acid content (Overby et al., '59b). Hydrogenated coconut oil was inactive (Overby et al., '59b).

The increased MR following the administration of thyroid has been reported to be decreased by the administration of fat (Abelin, '26; Abelin and Kursteiner, '28; Abelin et al., '30; Berg, '34) or linoleic acid (Keeser, '38). Fat feeding has also been reported to restore the loss of liver and muscle glycogen which results from thyroid administration (Abelin, '26; Abelin et al., '30; Zain, '36, '37).

Casein has been recognized as a poor source of antithyrotoxic activity (Tappan et al., '53; Graham et al., '53; Ershoff, '47; Dryden et al., '60; Overby et al., '59a), although increasing its dietary concentration (e.g., from 30 to 40%) may have a slight protective effect (Overby et al., '59c).[14,15] Soybean meal has been reported to vary in activity from poor (Tappan et al., '53; Stevens and Henderson, '58) to moderate (O'Dell et al., '55; Ershoff, '49) to good (Ershoff, '50). Soy protein has varied similarly (Graham et al., '53; Ershoff et al., '59),[16,17] and different brands of soy protein have given different responses in the same laboratory (Dryden et al., '60). Lactalbumin and fibrin were reported to have good activity (Overby et al., '59c), whereas gelatin was inactive

[8] Emerson, G. A., B. Esser and A. C. Page 1956 Nutritional studies with rats subjected to thyrotoxic stress. Federation Proc., *15*: 549 (abstract).

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

[10] See footnote 8.
[11] See footnote 9.
[12] See footnote 8.
[13] See footnote 9.
[14] See footnote 8.
[15] See footnote 9.
[16] See footnote 8.
[17] See footnote 9.

(O'Dell et al., '55). Amino acids were inactive (Dryden et al., '60) and an amino acid mixture which corresponded to the composition of liver residue was also inactive (Dryden et al., '59c).

Both reserpine and 2-aminoethylisothiouronium bromide (AET) have been reported to have moderate activity in growth and survival experiments (Overby and Fredrickson, '60a), but the response to reserpine has not been observed consistently, and Ershoff ('58) obtained less growth and a shorter survival in the presence of reserpine than in its absence. In large doses thiouracils depress the peripheral deiodination of thyroxine (Escobar and Morreale, '61) and inhibit the increased oxygen consumption which results from the administration of thyroxine and certain of its analogues (Andik et al., '49; Stasilli et al., '60).

Antibiotics have given variable results in different laboratories. Chlortetracycline and penicillin were reported to be inactive (Ershoff, '50; Dryden et al., '60) but both Meites ('52) and Overby et al. ('59a) obtained a growth response from procaine penicillin; no change in MR was reported originally (Meites and Ogle, '51), but some decrease in MR was reported for both procaine penicillin and chlortetracycline when the rats were not fasted prior to the determination (Vogel et al., '58). Neomycin has also been reported to give a growth response, whereas streptomycin had no effect (Meites, '52).

SUMMARY

(1) The antithyrotoxic activity of various dietary supplements was compared with a standard liver residue by an assay procedure in which weanling male rats were fed a purified 30% casein diet containing 0.1% of iodinated casein (1% thyroxine equivalent) with or without the test supplement for 19 to 28 days; metabolic rates were determined after 19 days and compared with the metabolic rate decreases produced by the inclusion of liver residue in the diet. The average metabolic rates which constituted the assay curve were 7.2 (liters of O_2 consumed per square meter body surface per hour) for the nonthyroidal basal group, 13.5 for the unprotected group fed 0.1% of iodinated casein, 10.2 when 5% of liver residue was included, and 8.2 for the 10% liver residue diet.

(2) Female rats were less satisfactory for the assay but responded to the test supplements like the males; the inhibition of the normal development of the ovaries and uterus produced by feeding iodinated casein was overcome by all those substances which effectively reduced the metabolic rate.

(3) A similar assay procedure in which a diet containing 0.8 mg/kg of triiodothyronine (T_3) was fed to weanling male rats for 16 to 28 days before determining the metabolic rate was less satisfactory than the use of iodinated casein because of the relatively high residual metabolic rate that remained in the presence of a large excess of the antithyrotoxic factor. In general, the same substances were active against both T_3 and iodinated casein, but the test with T_3 was more rigorous.

(4) The adrenal hypertrophy that resulted from the thyroid feeding was reduced by the various test substances to the same degree that these substances reduced the metabolic rate.

(5) The following substances were good sources of antithyrotoxic activity (equal to or better than 5% of liver residue; 50 units or more) by all the criteria studied: 10% of liver residue, 5% of liver residue fat, 1% of liver residue concentrate, 5% of hemoglobin, 10% of cottonseed meal or fermentation residue, 20% of lactalbumin or fibrin, and 0.2% of deoxycholic acid, dehydrocholic acid or sodium glycocholate. Moderate activity (approximately 35 units) was exhibited in all tests by 20% of soy protein and in some tests by 10% of distiller's solubles, 10% of egg yolk, 0.2% of cholic acid or 10% of brewer's yeast. The 6-propyl-thiouracil also had a moderate-to-good effect in reducing the increased metabolic rate produced by iodinated casein or T_3, but had little or no effect on growth. The following substances had little or no antithyrotoxic activity by any criterion: 10% of dried grass, whey, wheat germ, casein, dry fish solubles or corn oil, 20% of egg albumin or 1% of cholesterol. Lithocholic and hyodeoxycholic acids were also inactive in the standard metabolic rate assay procedure, as were 10% of lard,

20% of gelatin and a variety of miscellaneous substances. Procaine penicillin gave erratic results, but along with other antibiotics was generally inactive.

(6) No substance tested gave a good growth response without also reducing the metabolic rate.

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Reduction of Liver Xanthine Oxidase Activity and Iron Storage Proteins in Rats Fed Excess Zinc[1]

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Previous investigations (Cox and Harris, '60; Magee and Matrone, '60; McCall et al., '61) have demonstrated a reduction of liver iron in rats fed a diet containing excess zinc. Cox and Harris ('60) stated that the decrease of liver iron is an early manifestation of zinc toxicity, whereas a decrease of liver copper is a relatively late effect. Recently, Cox and Hale ('62) found a reduction of liver iron in swine fed excess zinc without a concomitant loss of liver copper.

This investigation was initiated to obtain additional information concerning the anomaly in iron metabolism during zinc toxicosis and, hence, to aid in the explanation of the mechanism. In this study, the activity of xanthine oxidase and the concentration of ferritin and hemosiderin in the liver of rats fed a high level of zinc were determined.

EXPERIMENTAL

The experimental procedure was conducted in the same manner as described in an earlier report from this laboratory (Cox and Harris, '60). Throughout the study, 0.4% of zinc (as zinc oxide) was used in the diet to produce the toxicosis. After the various dietary regimens were completed, the rats were killed and the liver was removed for analyses. For xanthine oxidase determination, the livers were placed immediately in cold, 0.039 M sodium-potassium phosphate buffer (pH 7.4) and after being chilled, were blotted, weighed, and homogenized. Xanthine oxidase activity was determined on the homogenates by a colorimetric assay (Litwack et al., '53). The method of Gabrio et al. ('53) was used for the quantitative fractionation of liver ferritin and hemosiderin. Iron content was measured by the method of Sideris ('42) and molybdenum by the thiocyanate technique as described by Sandell ('59).

RESULTS

Data demonstrating the effect of 0.4% of dietary zinc for 8 weeks, on liver ferritin, hemosiderin, and hemoglobin of male rats are presented in table 1. The concentration of both iron storage proteins was reduced ($P < 0.01$); however, the depletion of ferritin (80.7%) was greater than that of hemosiderin (66.6%). Of particular significance, a higher percentage of the total iron loss in the liver came from the ferritin fraction (77.2%) rather than hemosiderin (19.6%). The data also show that the relative distribution of storage iron between the iron proteins did not remain constant, the percentage of hemosiderin remained essentially the same, whereas that of ferritin decreased. As anticipated, the hemoglobin content in the liver decreased.

Data illustrating the effect of 0.4% of dietary zinc on rat liver xanthine oxidase activity are tabulated in table 2. The excess dietary zinc caused a reduction in activity, which occurred after feeding the diet a relatively short time. The data show that after 14 days, a loss in activity of approximately 50% occurred in the liver of the female rats. However, in the male rats, about 70 and 60% reduction in activity was noted after 4 and 7 days, respectively. Since it was demonstrated that the activity of xanthine oxidase was reduced, it seemed pertinent to ascertain

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TABLE 1

Excess dietary zinc and ferritin, hemosiderin, and hemoglobin in rat livers

Iron[1] as	Diet		Total Fe loss
	Basal (6)[2]	0.4% Zn (6)[2]	
			%
Total	178.9 ± 29.4[3]	46.5 ± 5.8[4]	
Ferritin	126.7 ± 21.4 (70.8)[5]	24.5 ± 3.8[4] (52.7)	77.2
Hemosiderin	38.9 ± 11.4 (21.7)	13.0 ± 2.9[4] (27.5)	19.6
Hemoglobin	13.4 ± 4.0	9.0 ± 1.6	3.3

[1] Micrograms per gram of wet weight of tissue. The average net weights of the liver for basal and zinc fed rats were 10.0 and 8.1 gm, respectively.
[2] Figure represents number of male animals fed diet for 8 weeks.
[3] Standard deviation.
[4] Significantly less than basal group, $P < 0.01$.
[5] Figures in parentheses show percentage of total iron as ferritin and hemosiderin.

TABLE 2

Excess dietary zinc and xanthine oxidase activity in rat livers

Days fed diet	Sex	Xanthine oxidase activity[1]	
		Basal[2]	0.4% Zn[2]
1	F	9.9	7.8
3	F	13.1	11.0
14	F	12.3	6.4
2	M	11.3	7.0
4	M	8.4	2.5
7	M	9.4	3.6

[1] Micromoles of xanthine disappearance per hour per gram of wet weight of tissue.
[2] Values are the average for two rats.

the status of molybdenum in the liver of rats fed excess zinc. Samples from an earlier investigation, in which rats were fed 0.4% of zinc for 8 weeks, were analyzed for molybdenum. No loss of molybdenum was noted; average liver molybdenum value for rats fed 0.4% of zinc was 1.1 ppm (dry weight basis) and for control rats, 1.2 ppm.

DISCUSSION

Granick ('54) stated that the ferritin fraction of liver, rather than of hemosiderin, is more readily available in the body for various metabolic processes of iron. Shoden et al. ('53) reported that iron is mobilized from both ferritin and hemosiderin, and they further stated that the compounds are functionally indistinguishable. They point out, however, that their study did not permit speculation concerning the intracellular exchange between the two compounds. Recently, Morgan ('61) reported that the reduction of storage iron in iron-depleted rats was accompanied by little change in the relative distribution of iron between ferritin and hemosiderin. In the present investigation, although both iron proteins were significantly reduced in the liver, the depletion of ferritin was greater than hemosiderin. It also was noted that a higher percentage of the total iron loss was the result of the loss of ferritin rather than hemosiderin. And, in contrast to the investigation of Morgan ('61), in which he found that the relative distribution of the total iron between the iron proteins remained essentially the same, our results indicate that hemosiderin remained the same but ferritin decreased. These data indicate, therefore, that the ferritin fraction was more labile than hemosiderin and was the major source of iron loss from the liver. However, as pointed out by Shoden et al. ('53), the role of any possible intracellular exchange between ferritin and hemosiderin could not be determined.

In an earlier investigation (Cox and Harris, '60) on zinc toxicosis in the rat, liver iron was reduced to a minimal value, after which no further depletion occurred. Underwood ('56) stated that iron reduction in tissues reaches a base line below which tissues would not release iron under any conditions of stress. Morgan ('61) noted that the complete removal of storage iron from the liver and spleen of iron-depleted rats was not achieved, but no definite lower limit of concentration could

be demonstrated. From these results and those of the present investigation, one may speculate that either the iron in the storage proteins is in a form in which it can not be further released or some mechanism responsible for the iron release has been destroyed.

The present investigation on the role of xanthine oxidase during zinc toxicosis was prompted by the report (Mazur et al., '58) which showed that xanthine oxidase participated in the *in vivo* liberation of liver ferritin iron to the circulation. It was thought that the action of zinc could possibly be one of increasing the activity of xanthine oxidase and consequently increase the rate of removal of iron from the liver. This apparently is not the case, however, since the activity of xanthine oxidase was reduced; in fact, the reduction was found after feeding the diet a relatively short time. In this respect, it has been shown (Cox and Harris, '60) that the loss of iron from the liver of rats fed excess zinc also occurs after feeding the diet a short time. Relevant to these results was the investigation (Kinney et al., '61) which showed that there were no concomitant changes in liver iron or iron absorption associated with decreased xanthine oxidase activity. Strohmeyer et al.[4] also failed to demonstrate a relationship between liver xanthine oxidase and iron metabolism.

Richert and Westerfeld ('54) reported that rats with an iron deficiency did not exhibit an altered liver xanthine oxidase. It could be concluded, therefore, that the reduced enzyme activity noted in the present investigation was not the result of the loss of liver iron which occurs during zinc toxicosis. On the other hand, a rapid loss of liver iron, such as that found in rats with zinc toxicosis, as compared with the relatively slow rate of iron loss of rats fed an iron-deficient diet may be important. The failure to observe a reduction in the molybdenum content in the liver of rats fed excess zinc suggests that the reduced enzyme activity was not due to a loss of this element. The early reduction of the activity of xanthine oxidase precludes the possibility of an effect of dietary protein intake; low protein intake was shown (Westerfeld and Richert, '50) to reduce liver xanthine oxidase.

SUMMARY

Experiments were made to study the effect of 0.4% of dietary zinc on the activity of xanthine oxidase and the content of ferritin and hemosiderin in the liver of rats.

The feeding of a high level of zinc produced a lowering of xanthine oxidase activity in the liver. The loss occurred after the rats had eaten the diet for a short time. Relevant to this result was the observation that liver molybdenum was not reduced in rats fed excess zinc.

Both iron storage proteins in the liver of rats fed excess zinc were reduced. The percentage loss of ferritin was greater than that of hemosiderin, and ferritin contributed a greater amount to the total iron loss from the liver. These results suggest that, under the conditions of the experiment, ferritin was more labile than hemosiderin.

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Effect of Methionine and Other Nitrogen Sources on Biochemical Processes in the Liver[1]

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The labile nature of liver protein was observed by Addis and associates ('36) who found that the loss of liver protein was high when rats were starved for two days. This decrease in nitrogen has been found to be a loss in actual liver cytoplasm (Kosterlitz, '44a). The loss of basophilic staining substance, presumably ribonucleic acid (RNA), and the increase of fat have also been reported by other investigators (Elman and Heifetz, '41; Elman, '43; Kosterlitz, '44b, '47; Allison et al., '56).

Liver deoxyribonucleic acid (DNA) concentration was found to increase after feeding a protein-free diet (Campbell and Kosterlitz, '47; Thomson et al., '53); however, no change was noted in the total liver DNA or the average DNA per nucleus (Campbell and Kosterlitz, '52). This report was in conflict with the data of Ely and Ross ('51) who found an increased amount in the average DNA content of the nucleus. Recently, Zigman and Allison ('59) observed that severe protein depletion produced by feeding a protein-free diet caused an increase of liver ribonuclease and a decrease of liver RNA-phosphorus. More recently, papers have appeared which demonstrate an increased synthesis of liver DNA in choline deficiency (Farish et al., '61) and in ethionine feeding (Stekol et al., '60). Similarly, Williams ('61) concluded that there was an increase in the number of cells per unit of weight of liver based on a DNA concentration increase of 1.5 to 1.8 times during protein deficiency.

The following studies were undertaken to determine some of the specific effects of methionine and other supplementations such as glycocyamine on metabolic processes in the liver. Particular emphasis was placed on protein anabolism as it may be connected with nucleic acid synthesis.

MATERIALS AND METHODS

One hundred weanling male Sprague-Dawley rats were divided into 10 groups after they were first paired with respect to body weight so that the initial weights of the dietary groups were the same. The groups were fed a basic diet containing 12% of casein (Allison et al., '54). The diets were supplemented with 0.7% of DL-methionine or 0.7% of DL-methionine plus 0.7% of glycocyamine. Previous studies have demonstrated that these amounts of methionine and glycocyamine are optimum for maintenance of adult rats and for growth in young rats fed a diet containing 12% of casein.[3] Glycine (1.28%), alanine (1.53%), and ammonium citrate (1.95%) used in isonitrogenous amounts compared with glycocyamine were also studied as single supplements and in combination with methionine. The rats were fed their respective diets for an 8-week period at the end of which time the animals were autopsied. In one series of experiments, S^{35}-L-methionine was injected intraperitoneally one hour before autopsy. In another series, liver slices were taken from each animal at the time of autopsy and incubated for two hours at 37°C in a Krebs-Ringer bicarbonate solution containing 100 mg/100 ml of glucose and 30 mg/100 ml of S^{35}-L-methionine. The livers were removed, rinsed several times in saline and an aliquot of approximately 500 mg of tissue was taken for digestion by a 1:1 mixture of concentrated H_2SO_4

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[1] This work was supported in part by grants from the United States Public Health Service 4341.

[2] Present address: Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri.

[3] Baron, H., and J. B. Allison 1954 Effect of methionine and glycocyamine on growth of rats. Federation Proc., *13*: 450 (abstract).

and concentrated HNO_3. The digest was then assayed for radioactive sulfur in a windowless proportional gas-flow counter.

The micro-Kjeldahl method (Pregl and Roth, '35) was used to measure the total protein content of liver, and liver lipid was determined gravimetrically after extraction with alcohol and ether, using a modified Soxhlet tube. The method of Schmidt and Thannhauser ('45) with slight modification, in conjunction with the phosphorus determination of Fiske and Subbarow ('25) was used for the measurement of the nucleic acids.

RESULTS AND DISCUSSION

The slope of the line obtained by plotting body weight gain against nitrogen intake has been related to protein quality (Allison et al., '59). Supplementation of a casein diet with either glycocyamine, glycine, alanine, or ammonium citrate decreased or had little effect on the efficiency of the dietary protein (fig. 1). However, supplementation of these diets with methionine increased the growth rate to a level higher than that of the casein-fed animals. This is in agreement with Baron ('58) who found that the addition of methionine to glycocyamine in suitable concentrations overcame to a large degree the growth inhibition caused by feeding glycocyamine. It was also shown by Allison ('56) and Hetzel[4] that methionine, when added to a protein-free diet, reduced the excretion of urea nitrogen. This observation suggests that amino acid is involved in the catabolism of the labile protein stores.

The livers of rats that were fed a diet containing 12% of casein and an excess amount of choline had a low protein and a high fat content (fig. 2). This type of

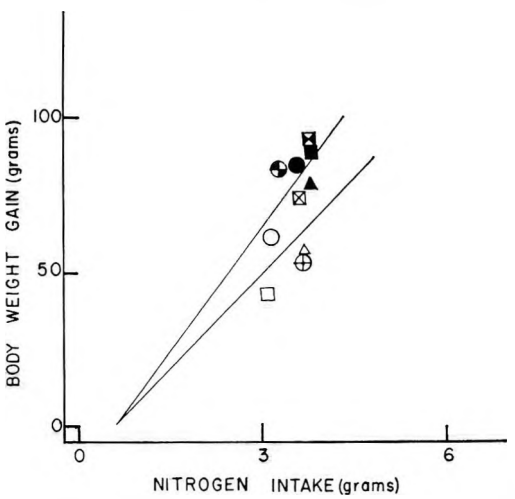

Fig. 1 The body weight gain plotted against nitrogen intake of rats fed casein diets supplemented with various nitrogen sources. ○ indicates casein diet; ⊕, casein plus ammonium citrate; □, casein plus glycocyamine; ⊠, casein plus glycine; and △, casein plus alanine. The symbols with any shaded area represent the addition of methionine to the diet.

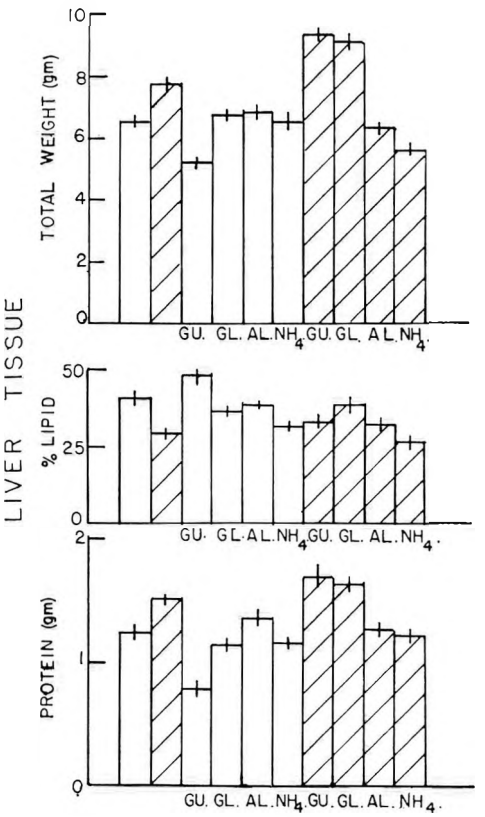

Fig. 2 Total liver weight, liver lipid per cent of dry weight, and total liver protein of rats fed casein diet and casein supplemented diets for 8 weeks. Standard error of the mean is shown by the perpendicular line to the top of the bar of each group. White bars indicate casein, and slanted lines, methionine supplementation to the diet. GU represents glycocyamine; GL, glycine; AL, alanine; and NH_4, ammonium citrate.

[4] Hetzel, C. A. 1957 Studies on protein depletion and methionine supplementation in the rat. Ph.D. Thesis, Rutgers University, New Brunswick, New Jersey.

liver was shown by Harper et al. ('50) to be associated with a reduced methionine intake. When the casein diet was supplemented with methionine, the protein level of the liver increased, whereas the lipid content decreased. The reverse effect was observed when the casein diet was supplemented with glycocyamine, indicating that methionine had been diverted into other pathways. The protein and lipid content of livers of rats receiving nonessential amino acids such as glycine or alanine were not significantly different from those of the casein controls.

Many of the amino acids serve special roles in the body in addition to their more common structural requirements in protein molecules. Methionine can function as a methylating agent in the formation of adrenaline (Keller et al., '50) and in the conversion of glycocyamine to creatine (du Vigneaud et al., '40, '41; Borsook and Dubnoff, '47). The data obtained on the incorporation of S^{35} from labeled methionine *in vitro* and *in vivo* by the liver of the rat clearly demonstrate the dual role of methionine. A high uptake of the S^{35} from labeled methionine was observed in livers of rats fed a casein diet that was low in sulfur amino acids (white bar, figure 3). This increase can be related to a decrease in amino acid pool size (Wannemacher, '61). The incorporation was further increased when rats were fed the casein diet supplemented with glycocyamine. Glycocyamine diverts some of the methionine into other pathways, which results in a methionine deficiency. When the casein diet supplemented with methionine was fed to rats, there was a significant decrease in the incorporation of S^{35} from labeled methionine by liver protein. Thus, this

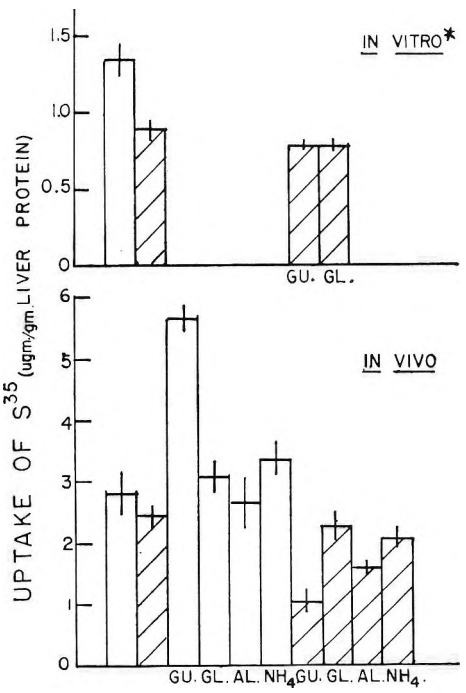

Fig. 3 The incorporation of S^{35} from labeled methionine into the liver protein of rats fed various diets for 8 weeks. Each value is given with the standard error of the mean. Symbols as in figure 2. *In vitro* (*) experiment was made using liver slices incubated for two hours at 37°C in a Krebs-Ringer bicarbonate solution containing 100 mg/100 ml of glucose and 30 mg/100 ml of S^{35}-L-methionine.

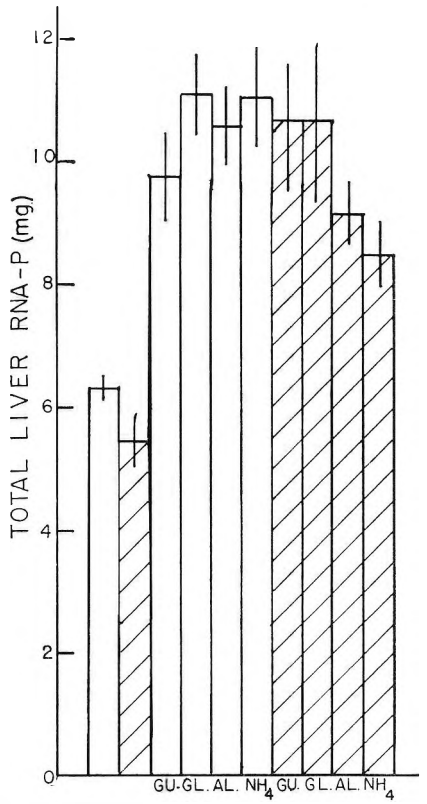

Fig. 4 Total liver ribonucleic acid phosphorus of rats fed various diets for 8 weeks. Each value is given with the standard error of the mean. Symbols as in figure 2.

study emphasized the dual role of methionine not only as an essential amino acid in the formation of tissue protein but also as an intermediate in biochemical reactions of the organism.

Allison et al. ('61) found that liver ribonuclease activity was increased and RNA was decreased at low nitrogen intake. When the nitrogen intake was increased, there was a sharp decrease in ribonuclease activity accompanied by an increase in liver protein. The data in figure 4 illustrate the increase in total liver RNA-phosphorus when the casein diet was supplemented with various nitrogen sources. A slight depression in RNA-phosphorus was noted when the casein diet was supplemented with methionine, but this was increased two-fold when the methionine was added in combination with glycocyamine.

The data in figure 5 illustrate the effect of various nitrogen supplements on the DNA-phosphorus content of the liver. A decrease in DNA or no change at all was observed when the casein was supplemented with glycine, alanine, glycocyamine or ammonium citrate. A slight increase was observed when the casein was supplemented with methionine. However, there was a marked increase in liver DNA when casein was supplemented with either methionine plus glycine (1.5-fold increase in liver DNA) or methionine plus glycocyamine (3.5-fold increase in liver DNA, figure 5). This increase would then be a reflection of an increase in cell number or nuclear size.

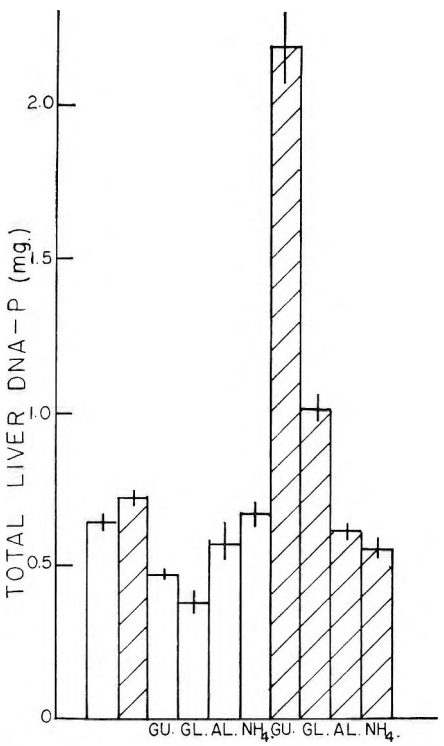

Fig. 5 Total liver deoxyribonucleic acid phosphorus of rats fed various diets for 8 weeks. Each value is given with the standard error of the mean. Symbols as in figure 2.

SUMMARY

When rats were fed diets containing 12% of casein supplemented with either glycocyamine or glycine, a slight decrease was observed in their growth rate. This decrease was overcome by the addition of methionine to the respective diets.

An increase was observed in the total liver protein and a corresponding decrease in liver fat when the rats were fed the casein diet supplemented with methionine. A decrease in liver protein and an increase in the percentage of liver fat was noted in rats being fed the diet supplemented with glycocyamine.

The incorporation of the S^{35} from labeled methionine into liver was reduced when the animals were fed the casein diet supplemented with methionine and was reduced further when the casein diet was supplemented with methionine plus glycocyamine or methionine plus glycine.

A working hypothesis was developed which emphasized the dual role of methionine in protein anabolism, first as an essential amino acid in the structure of tissue protein, and second as a methylating agent, possibly contributing to the energy requirements for synthesis.

ACKNOWLEDGMENT

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The Relative Roles of Vitamins, Protein, and the Salmonellosis Resistance Factor in the Natural Resistance of Mice to Salmonellosis[1]

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The supposition is often made that the nutrition of the host can affect its natural resistance to infection. Investigations directed toward an analysis of this supposed relationship, however, have yet to arrive at any consensus (*vide infra*). In the main such attempts at analysis have been based on the experimental manipulation of three categories of nutritional substances: vitamins, protein levels, and unidentified factors present in natural foodstuffs. This last category may need some prefatory comment.

Schneider and Webster ('45) found that mice consuming diets containing wheat survived *Salmonella enteritidis* infection in significantly higher frequency than those eating a "synthetic" diet. In subsequent publications (Schneider, '46a, '48, '49) the necessary genetic heterogeneity of the host population and polymorphic[3] character of the pathogen population for the maximal effect of diet were established. The resistance-promoting properties of wheat were traced by special assay procedures (Schneider and Zinder, '56) to a novel unidentified factor and concentrated a million-fold (Schneider, '56). This factor, designated as the Salmonellosis resistance factor (SRF),[4] enhances host resistance to *S. typhimurium* as well (Schneider, '46a). The SRF has no antibiotic properties as measured by conventional *in vitro* procedures, nor is it a growth factor for either mice or *Salmonella*.

While the effect of SRF supplementation in enhancing the natural resistance of mice to salmonellosis has been consistent, such has not been the case in studies on protein intake. Dubos and Schaedler ('58) and Schaedler and Dubos ('59), for example, found that increased protein intake *increased* the survival time of mice in tuberculosis and some other infections. Koerner ('49) obtained a similar result with rat tuberculosis. In contrast to these results, Smith and Chubb ('57) and Hill and Garren ('61) reported that increased dietary protein levels resulted in *decreased* survival times of chicks infected with *Salmonella gallinarum*. To complete the gamut of possible experiences, Ratcliffe and Merrick ('57) concluded that dietary protein levels had *no* effect on the susceptibility of rats or guinea pigs to tuberculosis, although with moderately virulent strains of the bacillus, the secondary lesions in the guinea pig healed more rapidly in animals receiving higher levels of protein.

As a nutritional category the vitamins, in their multiplicity, make impossible any general statement about their reported effect on resistance or susceptibility. Suffice to say that deficiencies of some vitamins have been reported to decrease resistance to some infections and increase resistance in others. Schneider ('46b) has reviewed the work in this area of investigation. In

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[2] This investigation was supported in part by a Special Fellowship (EF-12,964) from The National Institute of Allergy and Infectious Diseases, Public Health Service.
[3] This is also a kind of genetic heterogeneity, but one operationally arranged by bringing together, in the host, both virulent and avirulent genotypes of the pathogenic species. Since these two forms are indistinguishable by any known criteria, save the capacity to induce disease, the polymorphism thus arranged is more accurately labeled as a cryptic polymorphism.
[4] Wood, H. N., and H. A. Schneider 1957 Distribution, concentration and properties of *Salmonellosis* resistance factor (SRF). Federation Proc., *16:* 403 (abstract).

one instance (Hill and Garren, '55), the addition of large amounts of all the known vitamins to a diet already adequate in these substances increased the resistance of chicks to *S. gallinarum* infection.

The discordance of results of the studies with protein or vitamins might be taken as reflecting certain fundamental specificities of the particular pathogen or the particular host or their mutual combination. If this were true, even the hope of any meaningful generalizations would be remote indeed. On the other hand, there might well have been important and unanalyzed differences in some of the conditions inherent in the various studies which were responsible for the apparently conflicting results. For instance, the composition of the basal diet used by different investigators was not the same, and it is possible that resistance and susceptibility are influenced by interactions between dietary components with important consequences for analysis and interpretation. What might be true in one dietary context might not be so in another. In order to assess adequately these possibilities it becomes mandatory to assemble into one diet-infection model the three nutritional categories which have been reported to influence the outcome of an infection and to study their possible effects *simultaneously*. This report presents the results of such a study.

EXPERIMENTAL

The plan of this study was to examine the natural resistance of mice to salmonellosis as affected by three levels of protein and two levels of vitamin supplementation, with and without a source of SRF, in a factorially designed experiment with two replications. The 12 diets used are presented in table 1. The protein level was manipulated by varying the amount of casein to supply 5, 15, and 30% of protein. The level of cystine was kept proportional to the casein so that the amino acid balance remained constant at all levels of protein. The vitamin supplement at the lower level fed supplied all the known vitamins in amounts presumed adequate to meet the requirements of the mouse, a so-called "normal" intake. The high vitamin supplement was tenfold that of the lower. The crude SRF used was produced by microbial means on a chemically defined medium as described by Schneider and Wood[5] and Wood and Schneider.[6] The amount of SRF used was enough to protect 80% of the mice under the conditions of the bioassay (Schneider and Zinder, '56). The crude SRF concen-

[5] Schneider, H. A., and H. N. Wood 1959 The ecological origins of the *Salmonellosis* resistance factor (SRF). Federation Proc., *18:* 545 (abstract).

[6] Wood, H. N., and H. A. Schneider 1959 Properties of *Salmonellosis* resistance factor (SRF) produced by microbial means. Federation Proc., *18:* 552 (abstract).

TABLE 1

Experimental diets

Diet	A	B	C	D	E	F	G	H	I	J	K	L
Casein, vitamin-free	5	5	15	15	30	30	5	5	15	15	30	30
Salts W-2[1]	4	4	4	4	4	4	4	4	4	4	4	4
L-Cystine	0.1	0.1	0.3	0.3	0.6	0.6	0.1	0.1	0.3	0.3	0.6	0.6
Cottonseed oil, refined[2]	5	5	5	5	5	5	5	5	5	5	5	5
Glucose,[3] to	100	100	100	100	100	100	100	100	100	100	100	100
Vitamins	1[4]	1	1	1	1	1	10	10	10	10	10	10
Crude SRF[5]	0	+	0	+	0	+	0	+	0	+	0	+

[1] Schneider and Webster ('45).

[2] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

[3] Cerelose, Corn Products Company, Argo, Illinois.

[4] Supplies per 100 gm diet: vitamin A, 100 IU; vitamin D_2, 100 IU; and in mg, tocopheryl acetate, 8.0; menadione, 0.1; thiamine, 0.3; riboflavin, 0.8; pantothenic acid, 1.0; pyridoxine, 0.5; choline, 0.5; folic acid, 0.1; niacin, 1.0; inositol, 25.0; ascorbic acid, 100.0; biotin, 20.0 μg; vitamin B_{12}, 4.0 μg.

[5] Salmonellosis resistance factor, see text.

trate, as used here, contributed 2.6 gm of dried solids/kg of diet.

A strain of W-Swiss mice previously described (Schneider and Webster, '45) was used in this experiment. The program of exogamous breeding to maintain genetic heterogeneity (Schneider, '46) has been continued. In recent years the mouse colony has been maintained with a commercial pelleted mouse diet.[7] The mice are free of *Salmonella*.

The experiment was conducted in air-conditioned quarters at 80°F, 50% relative humidity, and with a 12-hour light day supplied by clock-controlled fluorescent lighting in a windowless room.

Three hundred female mice, 4 to 8 weeks of age, were weighed and divided by planned randomization into 12 groups of 25 mice each. Only one sex was used in order to reduce variance. (Schneider and Zinder, '56). No group contained siblings. The mice, averaging 20 gm in weight, were fed the experimental diets, lacking the SRF supplement, for two weeks. After this preliminary feeding period the 300 mice were individually caged, weighed, the SRF supplementation begun in the appropriate groups and each group of 25 divided into two sub-groups of 10 each plus 5 left as uninfected controls. After 4 days' acclimatization the mice were infected intraperitoneally by the double-strain inoculation procedure described by Schneider ('48). The dose was composed of 1,000 viable cells of avirulent *S. typhimurium* followed 48 hours later by 1,000 viable cells of virulent *S. typhimurium*.

After inoculation the disease was allowed to run its course for 30 days. By this time all deaths from the infection had ceased for 7 days and the survivors appeared to be fully recovered.[8]

The differences in survivorship were analyzed for significance by an analysis of variance.

RESULTS AND DISCUSSION

The results of the experiment are presented in tables 2 and 3 and the variance analysis in table 4. None of the 60 uninoculated control mice died; hence all deaths are attributable to salmonellosis. Body weight changes in the mice were slight during the two-week preliminary feeding period. With 5% of protein, average body weights remained stationary and gains of approximately 1 gm were made with the 15% and 30% protein diets.

[7] Old Guilford, Emory Morse Company, Guilford, Connecticut.

[8] Experience in this laboratory with this infection model over more than 20 years has shown that after 30 days, with the experimental conditions held constant, survivors of infection are in no further jeopardy from the disease.

TABLE 2

Survivorship results for replicated blocks of ten mice

Vitamin level	Protein level	SRF[1]	Diet	Replications		Treatment sum
				1	2	
	%					
"Normal"	5	0	A	6	6	12
		+	B	5	8	13
	15	0	C	4	4	8
		+	D	9	7	16
	30	0	E	4	3	7
		+	F	9	10	19
10 × "Normal"	5	0	G	3	4	7
		+	H	10	9	19
	15	0	I	7	5	12
		+	J	9	7	16
	30	0	K	4	5	9
		+	L	9	10	19
Replication sum				79	78	157

[1] Salmonellosis resistance factor; see text.

TABLE 3

Survivorship frequency differences by main effects

Treatment	Diets	Mice at risk	Survived	Survived	Difference
				%	%
Protein level, 5%	ABGH	80	51	63.8	
					1.2
Protein level, 15%	CDIJ	80	52	65.0	
					2.5
Protein level, 30%	EFKL	80	54	67.5	
"Normal" vitamins	ABC DEF	120	75	62.5	
					5.8
10 × "Normal" vitamins	GHI JKL	120	82	68.3	
No SRF[1]	ACE GIK	120	55	45.8	
					39.2
SRF	BDF HJL	120	102	85.0	

[1] Salmonellosis resistance factor; see text.

TABLE 4

Analysis of variance

Source	Degrees of freedom	Sum of squares	Mean square	F
Total	23	133.96	—	—
Protein levels	2	0.58	0.29	0.26
Linear	(1)	0.56	0.56	NS[1]
Deviation	(1)	0.02		NS
Vitamin levels	1	2.04	2.04	1.82
SRF[2]	1	92.03	92.04	82.17**
Protein levels × vitamin levels	2	0.58	0.29	0.26
Linear	(1)	0.06		NS
Deviation	(1)	0.52		NS
Protein levels × SRF	2	7.58	3.79	3.38
Linear	(1)	5.06	5.06	4.52
Deviation	(1)	2.52	2.52	2.25
Vitamin levels × SRF	1	1.04	1.04	0.93
Protein levels × vitamin levels × SRF	2	16.58	8.29	7.40**
Linear	(1)	10.57	10.57	9.30**
Deviation	(1)	6.02	6.02	5.37*
Duplicates	12	13.50	1.12	—

** Highly significant, $P < 0.01$.
* Significant, $P < 0.05$.
[1] Not significant.
[2] Salmonellosis resistance factor; see text.

These were statistically insignificant in the face of the variance encountered.

That nutritionally effected body weight changes have no necessary predictive value for the outcome of an infection was shown by Schneider and Webster ('45) and has been concurred in by Dubos and Schaedler ('58).

When the three nutritional categories were examined as separate factors, only crude SRF significantly affected the outcome of infection (table 4). The highly significant decrease in the number of deaths in those lots receiving SRF together with the lack of significance of the protein or vitamin levels alone, indicate that in a hierarchial arrangement of SRF, protein levels, and vitamin levels as used in this experiment, SRF must be ranked first in importance in increasing the resistance of mice to this infection.

While protein and vitamin levels failed to emerge as main effects in this study embracing an array of different nutritional contexts, it would be inaccurate to dismiss them as without meaning in this problem. Their relevance is indicated by the succeeding stages of the variance analysis.

As table 4 shows, SRF participates in a three-way interaction with vitamin and protein levels, although no two-way interactions were found at a *significant* level. These statistical statements merit some interpretive comment and further analysis if this three-way interaction is to be understood.

We can proceed with our analysis by fragmenting table 2 into halves, " 'normal' vitamins" and "10 × 'normal'." This will dispense with, for the moment, the vitamin aspect of the three-way interaction and we can now anticipate only two-way interactions, if such occur. The variance analysis of these halves of the experiment is presented in tables 5A and 5B.

TABLE 5A

Variance analysis of the "normal" vitamin level part of table 2

Source	Degrees of freedom	Sum of squares	Mean square	F
Total	11	60.25	—	—
SRF[1]	1	36.75	36.75	29.40**
Protein levels	2	0.50	0.25	NS [2]
SRF × protein levels	2	15.50	7.75	6.20*
Duplicates	6	7.50	1.25	

** Highly significant, $P < 0.01$.
* Significant, $P < 0.05$.
[1] Salmonellosis resistance factor; see text.
[2] Not significant.

TABLE 5B

Variance analysis of the "10 × normal" vitamin level part of table 2

Source	Degrees of freedom	Sum of squares	Mean square	F
Total	11	71.67	—	—
SRF[1]	1	56.33	56.33	56.33**
Protein levels	2	0.67	0.33	NS [2]
SRF × protein levels	2	8.67	4.33	4.33[2]
Duplicates	6	6.00	1.00	

** Highly significant, $P < 0.01$.
[1] Salmonellosis resistance factor; see text.
[2] Not significant.

On comparing table 5A with table 5B, only in the former, with "normal" vitamin levels, is there a significant interaction between SRF and protein levels. The SRF, of course, clearly emerges as a main effect in both tables, but multiplying the vitamin levels tenfold (table 5B) has lowered the SRF-protein interaction below statistical significance.

In table 5A, at "normal" vitamin levels, we are left with SRF and protein levels interacting. Protein levels still fail to exert an effect in their own right. The interaction, however, recommends a further penetration by analysis.

The final clue is now easily obtained. The survivorship data from the "normal" vitamins part of table 2 is graphed in figure 1.

Inspection of figure 1 clearly suggests that survivorship *does* vary with protein level, but it is obviously ambivalent. In the presence of SRF, increasing levels of protein *increased* survivorship in a linear way; in the absence of SRF, just the opposite occurs, increasing levels of protein *decreased* survivorship, also in a linear way. Thus the very direction of the survivorship differences attributable to protein was dependent upon SRF. This is a revealing insight into the interaction of SRF with protein.

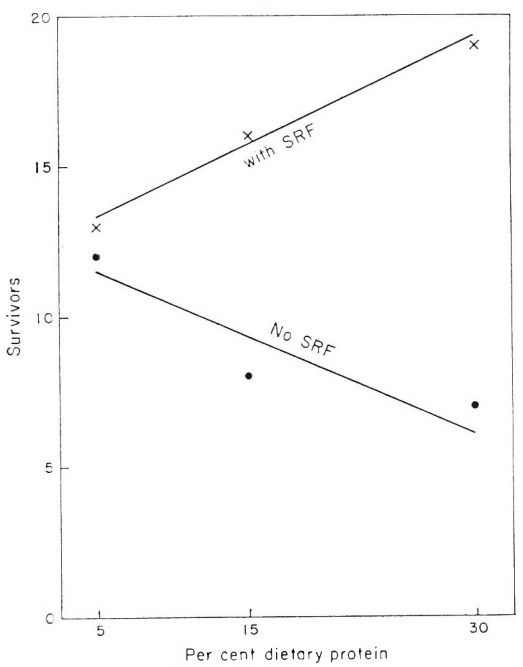

Fig. 1 Survivorship in mouse salmonellosis divergently affected by protein levels, depending on presence or absence of Salmonellosis resistance factor (SRF). ("Normal" vitamin levels throughout, 20 mice/test.)

But the graphic portrayal merely generates the hypothesis of this ambivalent effect. The crucial question is whether the relations observed in figure 1 are with certitude *separate*, *linear*, and *divergent* (have slopes of opposite sign). An orthogonal comparison using the data from the appropriate "normal" vitamin part of table 2 provides an affirmative answer to the question ($P < 0.01$). Since increasing levels of protein can exert these two precisely opposite effects on survivorship, depending on the presence or absence of SRF, it is obvious that no single declarative sentence can be formulated to give answer to the question "What is the effect of protein on natural resistance?" The question is now clearly naive and ignores a variable which is an integral determinant of the answer.

The foregoing analysis now makes possible an explication of the three-way, SRF × protein × vitamin, interaction by reconstruction as follows. At "normal" vitamin levels, SRF and protein levels are interacting, since survivorship frequencies vary in opposite ways with protein levels, depending on the presence or absence of SRF. This is a two-way interaction, SRF × protein level. But this two-way interaction is at some hazard, for now if vitamin levels are multiplied tenfold, this highly significant interaction is reduced to insignificance (see table 5B). Since the two-way interaction is thus nonadditively affected by a third parameter, vitamin levels, it follows that there exists a three-way interaction, SRF × protein × vitamins. This is precisely what the variance analysis of the complete experiment (table 4) presented.

To find, as has been found here, that protein level increases can generate, in one and the same infection model, divergent effects on survivorship appears to us to illuminate the ground for some of the conflicting results remarked earlier in this paper. Indeed such conflict could now be

interpreted as evidence for the supply or lack of supply of SRF or SRF-like substances in the various experimental diets and infections employed by the different investigators, all further perturbed by the vitamin levels, again variously chosen. The hope is thus reborn that some meaningful generalizations for the infection-nutrition problem may yet be arrived at, with the problem of specific infections (*Klebsiellae*, *Mycobacteriae*, *Brucellae*, and others) embraced by the specific SRF-like entity involved in each group of infections, and protein and vitamin levels participating as interactants in the ambivalent and more subtle ways we have observed here.

Finally, since SRF has been found in significant interaction with such classical nutritional entities as vitamins and proteins, SRF and hypothetical SRF-like entities appear to be a new kind of nutritional entity found in the natural world of foodstuffs and a legitimate subject matter for nutritional inquiry.

SUMMARY

The results and analysis of this factorial experiment can be summarized in the following set of statements, each of which can be asserted at the 99% confidence level.

In a factorial analysis of the relative roles of the Salmonellosis resistance factor (SRF), protein levels, and vitamin levels in survivorship in mouse salmonellosis, only SRF emerged with certitude as a resistance-promoting factor. At "normal" vitamin levels, increasing dietary protein levels ranging from 5 to 30% affected survivorship frequency linearly, but divergently: increasing survivorship obtained in the presence of SRF and decreasing survivorship in its absence. This two-way, SRF × protein, interaction was obliterated by increasing the vitamin levels tenfold. The consequence was a three-way interaction, SRF × protein × vitamins, the only other significant result to emerge from the factorial experiment.

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Impaired Pigmentation in Chinook Salmon Fed Diets Deficient in Essential Fatty Acids

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It has long been known that the fatty acids of fish are more unsaturated and have longer average chain lengths than do those of most warm blooded animals. However, the content of the so-called essential fatty acids in fish is low (Privett et al., '59, '60; Ahrens et al., '59; Stoffel and Ahren, '60; Thomasson, '53a, b).

There is some evidence to suggest that essential fatty acids are required by fish. For example, Mead et al. ('60), after injecting acetate-1-C^{14} into *Tilapia mossambica* and isolating and degrading the fatty acid, noted that the distribution of C^{14} was similar to that found in corresponding studies in mammals known to have an essential fatty acid requirement. They state that although small quantities of polyunsaturated acids can be synthesized from acetate, these are apparently not the higher essential fatty acids which must be formed from dietary linoleic acid. Klenk et al. ('60) showed that when liver slices from various fish were incubated with acetate-1-C^{14}, fragments produced by ozonolysis of the polyenoic acids had the label always predominating on the carboxyl side of the fatty acid. There was only low activity in malonic acid derived from the middle of the carbon chain and practically no activity in the propionic and caproic aldehydes derived from the methyl end of the fatty acid chain. These workers concluded that synthesis of C_{20} and C_{22} polyenoic acids in fish as in rats takes place essentially from exogenous precursors.

In this paper, data will be presented to show that when salmon fry are fed an essential fatty acid-free diet from the time of hatching, they undergo a change in color from their normal greenish-black to a light brown. This depigmentation may be one of the ways in which the salmon manifests a dermal syndrome in essential fatty acid deficiency.

EXPERIMENTAL

Five duplicate lots of 300 previously unfed chinook salmon (*Oncorhynchus tshawytscha*) fry were fed 5 different diets containing highly purified triglycerides or fatty acids[2] as the sole fat source. Fats used in each diet were: no. 1, no fat; no. 2, trilinolein; no. 3, triolein; no. 4, linolenic acid; no. 5, trilinolein and linolenic acid. All diets were held isocaloric at 348 Cal./100 gm of dry ingredients, assuming 9 Cal./gm of fat and 4 Cal./gm of sucrose or protein. Complete diet ingredients are listed in table 1.

To retard air oxidation of the highly purified synthetic fats used in the experiment, α-tocopherol was added each time a sealed vial of fat was opened. At the same time, vitamin D_3 was added to the fat mixture as this offered a convenient means of uniformly dispersing the small amount used. The fat-vitamin mixture was stored at 2°C under an atmosphere of nitrogen until diets were prepared.

Details of diet preparation are essentially as reported by Halver ('57) except that to minimize air oxidation, the fat-vitamin mixture was added in the last minutes of mixing after the diet was cool. Only sufficient diet for one week of feeding was prepared at a time. Between feedings, the diet was stored at 2°C in tightly closed jars.

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[2] Purchased from Hormel Institute, Austin, Minnesota.

TABLE 1

Diet composition[1]

Diet no.	1	2	3	4	5
Vitamin-free casein	37	37	37	37	37
Gelatin	12	12	12	12	12
Sucrose	37	34.8	34.8	36.8	34.8
Mineral supplement[2]	4	4	4	4	4
Vitamin supplement[3]	1	1	1	1	1
L-Cystine	0.5	0.5	0.5	0.5	0.5
L-Arginine	0.5	0.5	0.5	0.5	0.5
Trilinolein	—	1.0	—	—	1.0
Triolein	—	—	1.0	—	—
Linolenic acid	—	—	—	0.1	0.1
α-Cellulose	8	9.2	9.2	8.1	9.1
Water	200	200	200	200	200

[1] Ingredients except fat purchased from Nutritional Biochemicals, Cleveland, Ohio.
[2] Mineral mix USP VIII, no. 2 containing in addition the following (in mg): $ZnSO_4$, 3.0; CuCl, 0.1; $MnSO_4$, 0.8; KI, 0.15; $CoCl_2$, 1.0.
[3] Containing in mg: thiamine, 5; riboflavin, 20; pyridoxine, 5; choline chloride, 500; niacin, 75; calcium pantothenate, 50; *i*-inositol, 200; biotin, 0.5; folic acid, 1.5; ascorbic acid, 100; vitamin B_{12}, 0.01; menadione, 4; beta carotene, 2. For ease of diet preparation, a vitamin, amino acid, α-cellulose (6-gm) mixture was prepared in 100 × these amounts. After thorough blending, the mixture was stored at 2°C in closed containers until needed. α-Tocopherol, 40 mg and vitamin D_3, 0.005 mg, were mixed with the oil of diets 2 to 5 (see text); for diet 1, they were dispersed in 1 gm of α-cellulose.

To feed the diet, small chunks were forced through a garlic press and the worm-like extrusions cut off at a length of 2 to 3 mm. These cut extrusions were dropped in the water as rapidly as the fish would consume them. Feeding was continued until the fish began to reject the offerings. The fish were fed in this manner three times daily, 6 days a week.

Dead fish were removed as soon as observed and examined for injury or disease symptoms. Each lot was weighed at bi-weekly intervals by gathering all the fish into a net, allowing the excess water to drain for 10 seconds, then transferring the fish to a tared container of water and weighing to within 1 gm. With few exceptions, all the feeding and weighing was done by one individual. Precautions were exercised to avoid contact of diet or fish with the hands.

The fish were contained in screened, plastic coated, redwood hatchery troughs to which particulate-free well water was supplied at a rate of 2 gal (3.8 liters)/min. Water temperature throughout the experiment was maintained at 11° ± 1°C by an automatic temperature controlling device. Troughs were 4 feet long, 16 inches wide and 14 inches deep. Uniform lighting was provided by overhead fluorescent lights during the work day.

After 16 weeks of feeding, it was noted that the color of a large proportion of the fish not receiving trilinolein (namely, diets 1, 3, and 4), had turned from a normal greenish-black color to a light brown color. This condition persisted and appeared to become progressively more pronounced as the experiment was continued. At 24 weeks, a visual estimation of the amount of color change was made by counting the number of fish that could be ascribed to each of three arbitrary classifications: "light," "medium" and "dark."

To determine whether the normal dark color could be restored if trilinolein was included in the diet, the fish on each dietary regimen were then subdivided into two subgroups of approximately 200 fish each for further feeding. One group continued to be fed its original diet and the other group was fed a recovery diet containing 3% of trilinolein. The levels of sucrose and α-cellulose were adjusted so that the recovery diet would be isocaloric with the original diets. An effort was made to keep the color and weight distribution the same for the pairs of subgroups.

At bi-weekly intervals all groups were counted as "light," "medium" and "dark." To insure consistency of this subjective measurement, one person made all color estimations. The reproducibility of the estimate was determined by recounting

TABLE 2

Degree of pigmentation of chinook salmon with and without essential fatty acids[1]

	Diet 1 No fat		Diet 2 Trilinolein		Diet 3 Triolein		Diet 4 Linolenic acid		Diet 5 Trilinolein + linolenic acid	
August 17[2]										
Light	50		3		44		39		15	
Medium	29		11		31		34		16	
Dark	21		86		25		27		69	
					Recovery period					
	Original diet	Recovery diet	Original diet	Recovery diet	Original diet	Recovery diet	Original diet	Recovery diet	Original diet	Recovery diet
August 22[3]										
Light	42	42	3	3	23	27	52	41	3	1
Medium	34	35	20	14	38	42	28	37	7	7
Dark	24	23	77	83	39	31	20	22	90	92
August 31										
Light	18	11	2	1	16	15	7	7	2	1
Medium	56	61	10	12	65	57	56	56	15	15
Dark	21	26	86	85	17	25	35	37	80	80
Mortality	5	2	2	2	2	3	2	0	3	4
September 14										
Light	16	10	1	1	11	8	14	5	3	1
Medium	38	40	8	7	36	48	21	23	13	8
Dark	34	46	84	84	46	30	59	65	75	83
Mortality	12	4	7	8	7	14	6	7	9	18
September 28										
Light	12	3	0	0	5	2	6	4	1	1
Medium	26	34	4	4	37	27	28	22	14	10
Dark	47	57	85	83	46	54	56	67	72	77
Mortality	15	6	11	13	12	17	10	7	13	12
October 12										
Light	11	3	0	0	8	3	5	3	1	1
Medium	27	40	6	9	27	24	28	21	12	8
Dark	40	50	76	76	45	55	53	67	69	73
Mortality	22	7	18	15	20	18	14	9	18	18
October 26										
Light	3	0	0	0	4	4	2	0	0	0
Medium	20	12	7	5	25	22	9	10	6	6
Dark	47	76	73	71	46	52	70	80	73	71
Mortality	30	12	20	24	25	22	19	10	21	23

[1] Numbers represent percentage of starting population of each subgroup (August 17 numbers are percentage of population at that time).
[2] August 17 is the end of the initial 24-week feeding period.
[3] On August 22 each dietary group was divided into 2 subgroups of approximately 200 fish each. One subgroup (original) continued to be fed the original diet and the other (recovery) was fed a recovery diet containing 3% of trilinolein (see text). In the 5-day interim, all groups were fed their original diet.

TABLE 3

Reproducibility of estimation of degree of pigmentation

Group	No. fish	"Light" [1]	"Medium"	"Dark"
		% (*avg*)	% (*avg*)	% (*avg*)
1	139	5.4(1.76)[2]	25.8(3.46)	65.6(2.94)
2	152	2.5(0.30)	24.1(3.88)	73.5(3.69)

[1] Average of 4 different estimates over a three-day period.
[2] Standard deviation in parentheses.

TABLE 4

Average gains of chinook salmon fed fatty acid test diets

Diet no.	1 No fat		2 Trilinolein		3 Triolein		4 Linolenic acid		5 Trilinolein + linolenic acid	
Test period										
Avg wt, 24 weeks	2.42		2.74		2.53		3.27		2.83	
Avg wt, start	0.42		0.43		0.41		0.42		0.43	
Avg gain	2.00		2.31		2.12		2.85		2.40	
	Original diet	Recovery diet	Original diet	Recovery diet	Original diet	Recovery diet	Original diet	Recovery diet	Original diet	Recovery diet
Recovery period[1]										
Avg wt, 34 weeks	3.22	3.58	3.71	3.95	3.36	3.72	4.53	4.28	4.06	4.05
Avg wt, 24 weeks	2.44	2.41	2.67	2.60	2.54	2.55	3.24	3.28	2.69	2.69
Avg gain	0.78	1.17	1.04	1.35	0.82	1.17	1.29	1.00	1.37	1.36
Average gain 34 weeks	2.78		3.35		2.94		4.14		3.77	

[1] See footnote 3, table 2.
[2] Recovery diet containing 3% of trilinolein (see text).

two of the groups of fish four separate times during a three-day period (table 3).

The experiment was terminated at 34 weeks.

To help identify the pigment system involved, histologic observations[3] were made on biopsies of skin specimens. These were taken from the same pigmented areas of "light," "medium" and "dark" colored fish. The following routine stains were used: hematoxylin-phloxine; giemsa; toluidine blue buffered at pH 5. The following special stains were also used: luxol fast blue combined with periodic acid-Schiff (Hale et al., '60); Sudan black B (Gomori, '52a); silver-orcein-aniline blue (Humason and Lushbaugh, '60); silver safranine (Lillie, '54); and dopa oxidase (Gomori, '52b).

RESULTS AND DISCUSSION

Growth. Growth data presented in table 4 show that, when substituted isocalorically for sucrose in a fat-free basal ration, either linolenic acid or trilinolein (diets 2, 4 and 5) elicited an increased growth response, whereas triolein (diet 3) did not. This was particularly evident in the recovery phase of the experiment. During this period, the average gain of the fish fed the recovery diet (containing 3% of trilinolein) was nearly 50% greater than that of the fish that continued to be fed the fat-free and triolein diets (diets 1 and 3).

Pigmentation. Figure 1 shows a photograph of live fish representative of the three degrees of pigmentation recorded. This photograph shows the magnitude of the color changes involved. Table 2 clearly shows that at the start of the recovery period, those fish that had not received trilinolein in their diet (diets 1, 3 and 4) were distinctly lighter in color than those that had (diets 2 and 5). During the recovery experiment all groups darkened appreciably, including those not receiving trilinolein in the diet. However, those that received trilinolein appeared to have darkened more. The experimental

[3] The preparation of specimens for histochemical observations were made at the Division of Dermatology of the University of Oregon Medical School by Miss Doris Brophy to whom we are very grateful. We would also like to thank Dr. Walter C. Lobitz, Jr. of this Division for his assistance in interpretation of the slides.

Fig. 1 Photograph of live fish illustrating what is here being termed "medium," "dark" and "light" color. Relative size should be disregarded. The white spots are water surface reflections.

evidence, showing that linolenic acids was utilized for growth yet did not support normal pigmentation, is analogous to its failure to satisfy the complete essential fatty acid requirement of the rat (Aaes-Jorgenson, '61).

The loss of pigmentation of the fish while they were deficient in an essential fatty acid, followed by a restoration of pigment while still deficient, is not without parallel for other manifestations of the essential fatty acid deficiency syndrome. Dhopeshwarkar and Mead ('61), for example, found that guinea pigs that were made deficient in essential fatty acids exhibited an initial loss and then a regrowth of hair. Biochemically, it is well known that alternate patterns of synthesis of unsaturated fatty acids are more pronounced during essential fatty acid deficiency. For example, trienes are found to increase greatly, especially in heart and liver tissues (Rieckenhoff et al., '49). It is not inconceivable that such alternate synthesis can later alleviate in part the multiple manifestations of the essential fatty acid deficiency syndrome.

In a preliminary experiment using a population of chinook salmon that had previously been fed a diet containing 7% of corn oil and 2% of cod-liver oil for 6 weeks, no similar deficiency syndrome was observed after 38 weeks of feeding a "fat-free" diet. This diet was essentially the

same as that of diet 1 reported here, but contained white dextrin instead of sucrose. Extraction of lipids from the dextrin, casein and gelatin used, followed by gas chromatographic analysis of the fatty acid methyl esters, disclosed that the dextrin would contribute approximately 0.06% of linoleic acid (dry weight basis) to the diet. Since the weight of these fish increased fivefold while they were being fed this "fat-free" diet, it appears that salmon either have the ability to guard tenaciously their essential fatty acid stores or to have a very low requirement for them or both.

That Kelly et al. ('58) did not observe this depigmentation process in the common mullet (*Mugil cephalis*) may mean that the essential fatty acid stores present in their fish (before feeding was begun) was sufficiently large to prevent the appearance of this syndrome. It is well known that it is very difficult to establish an essential fatty acid deficiency in adult animals.

Microscopic examination of live fish at magnifications up to 40 ×, showed that the dark appearance of the skin of normal salmon was mainly due to a large number of small black dots scattered over the dorsa and sides of the fish and in even rows on the fins. These dots were normally aggregated into clusters to make up the large spots seen on the upper portion of the fish. Examination of experimental fish in this manner showed that the number of pigmented dots distributed throughout the skin decreased very substantially in going from a "dark" to a "medium" to a "light" color. When the fish were killed by immersing them in formalin, all other colors faded except the black dots and the silver sheen, leaving a "black on white" picture. Once again, the "light," "medium" and "dark" fish could be easily distinguished.

Histochemical tests performed on the skin of these fish gave evidence that the pigmented dots involved in the depigmentation process were made by melanin-producing cells and that the dark pigment in question was melanin.

A depigmentation phenomenon was also observed by Basnayake and Sinclair ('56), who studied the effect of essential fatty acid deficiency on the hooded rat. They found that the black hood hair of this rat became brown after about 20 weeks of feeding a diet deficient in essential fatty acids. They state, "Since small tracts of new jet-black hair are observed to grow against the general brown background, there is no intrinsic local inability to form the usual black pigment but merely a slowing of the process. After therapy with essential fatty acids, a very prominent sign is the rapid growth of jet-black hair replacing the brown. The deficient animals replace hair poorly (Rokonnes, '53). The brown hairs may therefore owe their color to fading of the original black pigment."

Since an essential fatty acid deficiency can produce a dermal depigmentation in species as widely divergent as rats and fish, it appears that essential fatty acids are of fundamental importance in dermal pigmentation.

SUMMARY

1. A marked depigmentation was observed in the skin of chinook salmon fry fed a fat-free diet since hatching. A similar depigmentation was observed when triolein or linolenic acid was included in the diet, but depigmentation was largely prevented by the inclusion of trilinolein. Depigmentation became apparent after 16 weeks of feeding and reached a maximum in about 24 weeks.

2. General repigmentation occurred during a recovery experiment and appeared to be more pronounced and more rapid in subgroups fed a diet containing 3% of trilinolein than in the subgroups continued with their original diet.

3. Histochemical tests suggest that the depigmentation process involves melanin.

4. Trilinolein or linolenic acid, or both, elicited a positive growth response in chinook salmon fry when substituted isocalorically for sucrose in a fat-free ration, but triolein did not.

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Influence of Pyridoxine and Dietary Fat on the Distribution of Serum Fatty Acids in Dogs[1,2]

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Witten and Holman ('52) noted that rats synthesize arachidonic acid from linoleate and hexaenoic acid from linolenate, which conversions were stimulated by the presence of pyridoxine. Kirschman and Coniglio ('61), on the other hand, indicated that pyridoxine was not specifically concerned with the conversion of linoleic acid to arachidonic acid, but "may affect in a general manner the metabolism of the saturated and monounsaturated as well as polyunsaturated fatty acids." Greenberg and Moon ('61) observed that the presence or absence of pyridoxine in the diet did not influence the amount of arachidonic acid in the blood when fat-deficient monkeys were fed corn oil. The composition of tissue fat in pyridoxine deficiency has been studied by various workers (Williams et al., '59, '61; Johnston, et al., '61; Swell et al., '61), but no clear-cut conclusions have been drawn concerning a specific role for vitamin B_6 in the metabolism of the polyunsaturated fatty acids. In the attempt to delineate a possible function of vitamin B_6 in relation to the metabolism of essential fatty acids, it seemed pertinent to investigate the influence of dietary pyridoxine on the fatty acid distribution in blood serum of dogs maintained with different dietary fats.

MATERIALS AND METHODS

Six Beagles about 1.5 years old, two German short-hair puppies and 6 mongrel puppies were fed diets with or without fat containing 20% of the calories as protein. In the vitamin B_6-deficient diet, vitamin-free casein[4] was used as the source of protein. In the diets containing vitamin B_6, skim milk powder was used. When fat was used it constituted 15% of the calories, the remaining calories being supplied by sucrose. The adult animals were fed 100 Cal. per kg per day and the puppies 150 Cal. per kg per day. The mother of 4 mongrel puppies received a fat-free diet beginning 5 days postpartum. After weaning, three weeks later, the puppies were continued on a fat-free regimen. Cellulose[5] was added to increase the bulk of the food, and minerals were supplied as bone ash and salt mixture. Each day's diet was supplemented with a multivitamin preparation containing vitamins A, D and E,[6] and for each 100 Cal. of the food were added: thiamine, 0.1 mg; riboflavin, 0.1 mg; niacin, 2 mg; and Ca pantothenate, 0.6 mg. In an attempt to reduce vitamin B_6 activity as much as possible, deoxypyridoxine[7] in the amount of 1 mg per kg per day was added to the diet of some of the animals for short periods of time.

Blood was drawn from the femoral artery after an overnight fast. Total cholesterol was determined according to the method of Sperry and Brand ('43). The total fatty acids of serum were prepared by the procedure routinely used in this laboratory (Wiese and Hansen, '53). For gas chromatography the esters were prepared by methylation with 2% sulfuric

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[4] Nutritional Biochemicals Corporation, Cleveland.
[5] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[6] Vi-Penta No. 2, Roche Laboratories, Inc., Nutley, New Jersey.
[7] See footnote 4.

TABLE 1

Weight, hemoglobin, hematocrit, total cholesterol, total fatty acids and relative distribution of fatty acids in blood serum in dog no. 2 receiving safflower oil in diet with and without pyridoxine

Date	Dietary fat	Vitamin B_6	Wt	Hemoglobin	Hematocrit	Cholesterol	Total fatty acids	Fatty acids as % of total fatty acids[1]						
								16	16:1	18	18:1	18:2	20:3	20:4
			kg	*gm/100 ml*	*%*	*mg/100 ml*								
7-21-61	Safflower oil	normal				55	312	7.2	1.1	18.0	6.5	41.6		22.4
9-15-61	Safflower oil	low												
10-5-61	Safflower oil	low	6.20			80	210	8.3	0.7	16.7	6.4	35.6		26.2
10-31-61	Safflower oil	low	6.01			71	281	9.9	2.2	16.8	7.0	41.9		17.5
11-15-61	Safflower oil	low	5.55			81	260	5.8	1.3	16.9	9.2	44.9		19.8
12-6-61	Safflower oil	low	6.26			62	250	11.5	1.8	14.4	10.2	42.7		16.9
12-27-61	Safflower oil	low	6.36	5.5	19	53	239	10.0	2.5	13.7	8.7	45.7		16.8
1-3-62	Safflower oil	pyridoxine added	6.46			63	275	11.5	0.9	17.4	6.1	46.1		15.1
1-11-62	Safflower oil	pyridoxine added	6.58			63	281	10.3	1.6	18.7	8.6	39.9		17.3
1-19-62	Safflower oil	pyridoxine added	6.70	6.3	29	71	257	11.8	0.8	17.3	6.6	44.5		16.5
1-30-62	Safflower oil	pyridoxine added	6.70			61	256	8.6	1.6	15.8	8.4	43.2		19.4
2-10-62	Safflower oil	pyridoxine added	6.63				285	9.4	1.0	20.0	5.2	40.3		22.8
2-21-62	Safflower oil	pyridoxine added	6.76	11.4	34		338	12.6	1.4	17.0	6.5	40.4		18.1

[1] The first figure represents the number of carbon atoms, the second the number of double bonds.

acid in absolute methyl alcohol. The determination of the distribution of the fatty acids was carried out with the Beckman GC-2A gas chromatograph using a 6-foot diethylene glycol succinate column at a temperature of 210 C and with helium as the carrier gas.

EXPERIMENTAL PROCEDURE AND RESULTS

The 6 adult Beagles were given diets varying both in fat and vitamin B_6 content.

Dog no. 1. To be certain that deoxypyridoxine in the amounts used acted as an antivitamin, and not as toxic agent, one animal received a diet containing corn oil, vitamin-free casein with deoxypyridoxine and at the same time pyridoxine was given at the rate of 3 mg per day. This animal's condition remained excellent at all times. No skin lesions developed and there was no anemia. After about two months on deoxypyridoxine the drug was discontinued. No significant changes in the amount of total cholesterol, total fatty acids or the relative distribution of the fatty acids in serum were observed during the 5 months of observation.

Dog no. 2. This animal was given the diet low in vitamin B_6 plus safflower oil. After about three months, hypochromic anemia developed. A brownish discoloration of the skin was noted with some loss of hair and the whiskers became slightly curly. The addition of pyridoxine (1 mg per kg per day) affected a prompt restitution of the skin to normal and within two months the anemia was corrected. The total cholesterol, the total fatty acids of the serum did not show consistent changes during this period although there was a trend for the 20:4 fatty acid[8] to decrease with low vitamin B_6 intake with a slight concomitant increase in the 18:1 fatty acid. These data are summarized in table 1.

Dog no. 3. The third adult animal received the same diet (low in vitamin B_6 plus corn oil) and in addition was given deoxypyridoxine. The sequence of events was the same as with the previous animal with respect to clinical manifestations, anemia and the serum lipid values except

[8] The first figure represents the number of carbon atoms, the second the number of double bonds.

the trends noted with the 20:4 and 18:1 fatty acids were not observed.

Dog no. 4. This adult Beagle was fed a diet low in vitamin B_6 and was given deoxypyridoxine for one month. Fifteen per cent of the calories were supplied as hydrogenated coconut fat containing only minute amounts of linoleic acid. The animal's condition deteriorated rapidly, with death occurring after two months. Serum lipid studies revealed the total fatty acids to be lower than normal with decreases in linoleic and arachidonic acids and increases in monoenoic and eicosatrienoic acids which features are characteristic of fat deficiency resulting from the low intake of linoleic acid. It was the additional deficiency of pyridoxine that probably caused the rapid deterioration and death of the animal.

Dogs no. 5 and 6. Inasmuch as the combined deficiency state (linoleic acid and vitamin B_6) apparently was lethal, at the beginning of the period of observation it was decided to use diets that were deficient in only one respect. Hence, the fifth adult Beagle received a diet which, at first, contained hydrogenated coconut oil, but later no fat. After fat deficiency signs became apparent the diet low in vitamin B_6 was given, but in order to accelerate the vitamin B_6 deficiency state, the animal was given deoxypyridoxine for a one-week period. The sixth animal was fed the diet low in pyridoxine and for a two-month period deoxypyridoxine was given; then the low fat was used for an additional month. The double deficiency state (linoleic acid and vitamin B_6) proved to be very hard on these animals. They appeared extremely weak, lost much of their hair, the whiskers became curly and they became inactive and appeared to have no initiative (see figs. 1 and 2). To prevent imminent death, it was decided to supplement the diets — first with corn oil and with pyridoxine. The serum fatty acid patterns changed typically and rapidly following the addition of the vegetable oil containing linoleic acid; however, no effect on the serum fatty acids could be attributed to the presence or absence of pyridoxine in the diet (figs. 3 and 4).

Inasmuch as Wiese et al. ('62) showed that growth is a most important factor for the production of fat deficiency symptoms, it was suggested that the lack of effect of pyridoxine on the serum lipids in the foregoing experiments might have depended upon the lack of growth in the adult animals. To evaluate this concept the same type of experiment was repeated with growing puppies.

Dogs no. 7 and 8. Two mongrel, littermate puppies at two months of age were first fed the fat-free diet containing no vitamin B_6. After one month the skin began to show typical scaliness, and the whiskers became curly. There was slight anemia in both animals. The puppies then were given safflower oil and to one, pyridoxine also was given. Whereas the fat deficiency pattern of the serum fatty acids immediately changed to a normal distribution, the presence or absence of pyridoxine had no influence on the level of arachidonic acid of the serum (table 2).

Dogs no. 9, 10, 11 and 12. The same procedure was repeated with 4 mongrel, littermate puppies, the only difference being that the animals were only 5 days old at the beginning of the study, the dam of the puppies having been fed a fat-free diet. After weaning, the same low-fat regimen was followed with the puppies. At the age of 6 weeks all 4 puppies were given diets low in pyridoxine as well as low in fat. Weight increase was satisfactory, but at about two months of age the scaliness of the skin, typical of fat deficiency, appeared. Two weeks later hypochromic anemia was noted. At this point all 4 pupplies received safflower oil and two of the subjects were given pyridoxine in addition. The change in the distribution of the fatty acids in the serum took place rapidly and in a similar manner in all animals, regardless of the presence or absence of pyridoxine in the diet (table 2).

Dogs no. 13 and 14. To test the influence of pyridoxine deficiency under more extreme circumstances, two littermate, German short-hair puppies from the age of three months were given a low-fat diet and from 3.5 months the low pyridoxine-low fat diet. To one animal deoxypyridoxine also was given for a period of 9.5 weeks. Both animals grew rapidly, were lively and vigorous. They showed only slight scaliness of the skin but a pro-

Fig. 1 Curly whiskers and "sad look" of puppy fed diet deficient in fat (linoleic acid) and pyridoxine (dog no. 6).

Fig. 2 Appearance of animals fed diet deficient in both linoleic acid and pyridoxine (dogs no. 5 and 6).

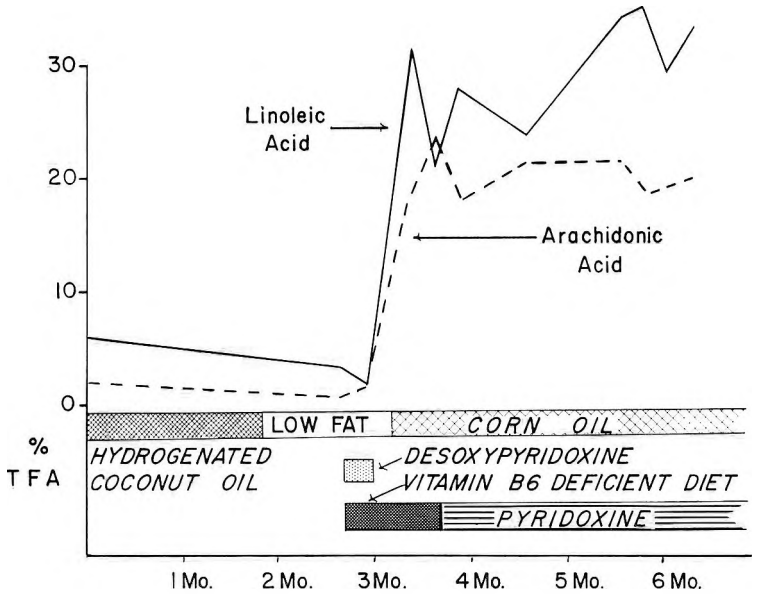

Fig. 3 Linoleic acid (———) and arachidonic acid (— — —) in blood serum as influenced by changes in diet (dog no. 5).

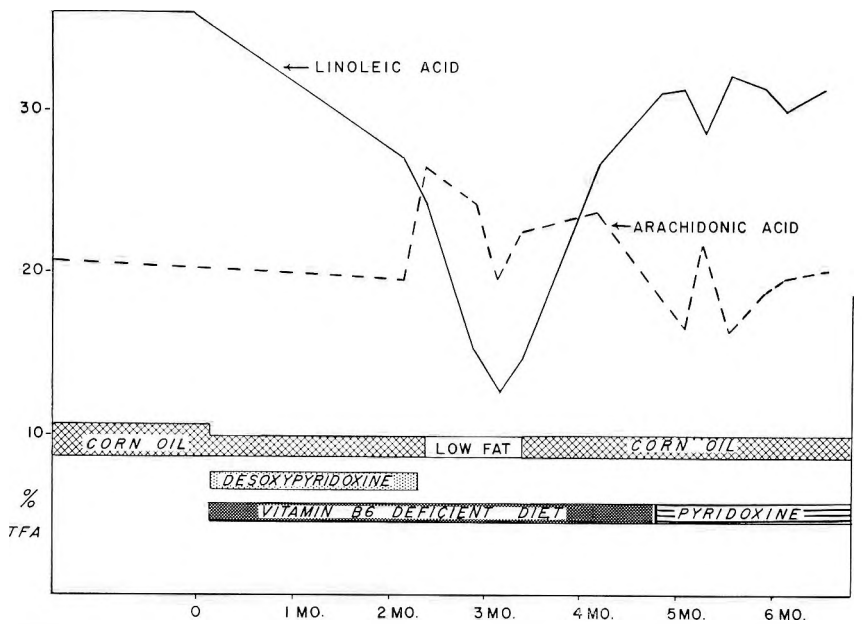

Fig. 4 Linoleic acid (———) and arachidonic acid (— — —) in blood serum as influenced by changes in diet (dog no. 6).

nounced fat deficiency pattern in the serum fatty acids. Both animals were then given safflower oil. Dog no. 13 received deoxypyridoxine as well as the diet low in vitamin B_6 for 13.5 weeks, whereas dog no. 14 was given the pyridoxine supplement for a 5-week period. In both animals the linoleic acid of the serum increased at about the same rate, but in the animal fed the pyridoxine-deficient diet plus deoxypyridoxine the increase in the relative content of arachidonic acid was slightly slower than in the littermate that received pyridoxine (fig. 5).

TABLE 2

Relative distribution of fatty acids in blood serum in puppies fed a low-fat diet and following the addition of safflower oil with or without pyridoxine[1]

Dog no.	Diet containing		Duration	Fatty acids as % of total fatty acids[2]						
	Linoleate	Vitamin B_6		16	16:1	18	18:1	18:2	20:3	20:4
			weeks							
7	—	—	4	17.8	8.1	10.1	37.0	4.5	12.8	8.3
	+	+	3	12.6	2.7	15.5	9.3	38.3	—	19.7
8	—	—	4	19.8	7.4	9.6	40.3	3.3	13.9	3.9
	+	—	3	13.1	1.8	13.6	11.1	29.9	2.3	22.3
9	—	—	4.5	16.2	6.3	12.3	39.6	6.3	12.3	3.5
	+	—	1.5	12.5	1.4	15.0	11.9	33.4	1.9	16.8
10	—	—	4.5	16.5	6.3	11.7	42.2	4.8	9.9	6.5
	+	—	1.5	11.3	1.5	13.7	13.8	35.8	0.4	17.7
11	—	—	4.5	14.6	6.1	9.8	42.3	3.6	16.7	4.7
	+	+	1.5	13.2	1.7	14.2	14.6	32.8	0.4	14.2
12	—	—	4.5	17.2	7.6	11.6	42.7	5.3	8.5	5.0
	+	+	1.5	13.5	1.9	15.1	13.5	29.4	0.5	18.9

[1] Dogs no. 7 and 8 are littermates; and dogs nos. 9–12 are littermates.
[2] The first figure represents the number of carbon atoms, the second the number of double bonds.

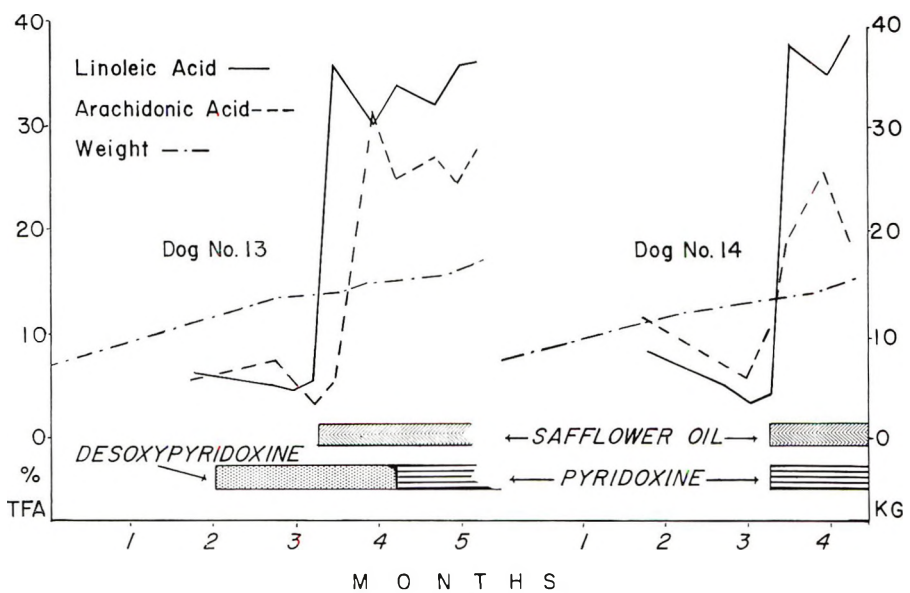

Fig. 5 Weight (— · —) and relative percentage of linoleic acid (———) and arachidonic acid (— — —) in blood serum in relation to diet (dogs no. 13 and 14).

DISCUSSION

In this study it was found that dogs with vitamin B_6 deficiency in addition to the previously described signs of hypochromic anemia, lack of initiative and hair loss (Street et al., '41; Emerson, '54; Hawkins, '55) rather consistently also developed curliness of the whiskers. In combination with fat deficiency, vitamin B_6 deficiency proved to be such a severe condition as to lead to a critical state or even early death. Despite the low dietary intake of vitamin B_6 there appeared to be no difficulty for the animals to synthesize arachidonate following the addition of linoleate to the diet. It might be speculated that the arachidonate could come from stores in the liver or other tissues which had been set free when the diet contained linoleic acid; however, evidence for this possibility is lacking. More probably the arachidonic acid is newly synthesized from the linoleic acid of the diet.

Inasmuch as vitamin B_6 has many functions and lack of this vitamin might influence many metabolic reactions, there seems to be no doubt that deficiency of this vitamin could be severe enough to prevent normal intracellular reactions. It was quite clearly shown in this study that the low dietary levels of pyridoxine did not influence the synthesis of arachidonic acid from linoleic acid. In one rapidly growing puppy, some delay in the increase of arachidonic acid level in serum was noted compared with that of the littermate receiving pyridoxine in the diet; however, the degree of increase was the same in both animals.

SUMMARY

Studies were performed concerning the relative distribution of fatty acids in the blood serum of 6 adult Beagle dogs, two German short-hair puppies and 6 mongrel puppies that received diets deficient in linoleic acid or vitamin B_6 or both. The presence or absence of linoleic acid caused marked changes in the fatty acid spectrum in the serum, whereas the presence or absence of pyridoxine in the diet did not influence the relative content of arachidonic acid in the blood serum. With two rapidly growing young animals (the German short-hair puppies) however, it was noted that arachidonic acid increased somewhat more rapidly in the serum of the animal receiving pyridoxine in the diet than in the littermate fed a diet without pyridoxine, although the magnitude of change was about the same. Apparently the conversion of linoleic acid to arachidonic acid is possible without the presence of vitamin B_6 in the diet.

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Iron Deficiency Studies in Chicks Using Treated Isolated Soybean Protein Diets

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Reid et al. ('56) showed that the molybdenum in isolated soybean protein was unavailable to the chick and that by use of this protein the dietary requirement was unfulfilled. Subsequently, Morrison and Sarett ('58), and others, reported that the chick's requirement for zinc is greater when fed a soybean protein diet than when given a purified casein diet. Kratzer et al. ('59) observed that the disodium dihydrogen salt of ethylenediaminetetraacetic acid (Na_2EDTA) decreased the poult's requirement for zinc when it was included in a diet containing isolated soybean protein. Scott and Zeigler ('60) confirmed this finding with chicks fed diets containing isolated soybean protein, and Lease et al. ('60) obtained comparable results by feeding chicks diets containing sesame seed oil meal. Davis et al. ('61) also reported that Na_2EDTA reduced the chicks' requirement for zinc when isolated soybean protein served as the source of protein in the diet. In addition, these authors reported that this protein reduces the availability to chicks of manganese and copper. In the case of zinc, manganese and copper, Na_2EDTA overcame the effect of soybean protein, presumably by removing the trace elements and thereby rendering them soluble (Chaberek and Martell, '59) and absorbable.

The work presented in this report was undertaken to obtain further information on the iron requirement of the chick by using a basal diet containing isolated soybean protein treated with Na_2EDTA in an attempt to reduce its iron content and thereby enhance the effects of iron deficiency. The results of this work together with the results of the analysis of untreated and treated soybean protein for iron and other mineral elements are presented in this report.

EXPERIMENTAL

On hatching, 5 male and 5 female one-day-old New Hampshire chicks per lot were selected by weight for experiments 1 and 2. Ten, one-day-old White Plymouth Rock male chicks per lot were selected for experiment 3. The chicks were identified with numbered wingbands and weighed individually at the start and weekly thereafter. They were housed in galvanized, electrically heated battery brooders with raised wire-mesh floors. All parts of the batteries were coated with an epoxy resin to prevent trace mineral contamination. The duration of each experiment was 4 weeks. Graded levels of iron were fed with the treated isolated soybean protein[1] basal diet in experiment 1 and graded amounts of both iron and copper were fed with this diet in experiment 2. The chicks in experiment 3 were fed treated isolated soybean protein and dried skim milk basal diets containing graded levels of iron. The composition of the basal diets is presented in table 1. Feed and distilled water were provided ad libitum.

The soybean protein was treated by suspending it in tap water at the rate of 454 gm of protein per 3.8 liters. The slurry was heated to 50°C with steam, and adjusted to pH 4.3, the isoelectric point of the protein, with sodium hydroxide. The Na_2EDTA was added at the level of 0.5% of the amount of the protein, and stirred for 30 minutes. The slurry was then allowed to settle, and the supernatant was syphoned off. The soybean protein used

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

TABLE 1

Composition of basal diets

Ingredient	Isolated soybean protein diet[1]	Dried skim milk diet[2]
	%	%
Protein source	25.0	64.0
Soybean oil	3.0	3.0
Vitamin mix[3]	3.15	3.15
Mineral mix[4]	2.27	2.27
DL-Methionine	0.5	0.3
Glycine	0.3	0.5
L-Arginine	0.0	0.5
Dicalcium phosphate	2.0	0.0
Calcium carbonate	2.5	1.77
Cellulose[5]	5.0 and 2.0[6]	2.0
Butylated hydroxytoluene	0.025	0.025
Cornstarch	56.28	22.5

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

[2] Dried skim milk, Foremost Dairies, Inc., San Francisco, California.

[3] When included in the diet at a level of 3.15% the vitamin mixture contributed the following vitamins in amounts per kg of diet: riboflavin, 10 mg; thiamine·HCl, 10 mg; pyridoxine·HCl, 10 mg; Ca pantothenate, 30 mg; niacin, 100 mg; folic acid, 5 mg; 2-methyl-1,4-naphthoquinone, 10 mg; vitamin A (20,000 IU/gm), 0.5 gm; vitamin D_3 (1,500 ICU/gm), 1.0 gm; vitamin E concentrate (44 IU/gm), 2.0 gm; choline chloride (25%), 2,000 mg; biotin, 0.2 mg; and vitamin B_{12}, 10.0 μg.

[4] When included in the diet at a level of 2.34% the mineral mixture contributed the following minerals in grams per kg of diet: NaCl, 10.0; $MnSO_4 \cdot H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.65; $CuSO_4 \cdot 5H_2O$, 0.08; ZnO, 0.07; $Co(CH_3COO)_2 \cdot 4H_2O$, 0.02; KI, 0.01; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.25; $MgSO_4 \cdot 7H_2O$, 4.0; KCl, 3.0; K_2HPO_4, 5.0; $Na_2MoO_4 \cdot 2H_2O$, 0.01.

[5] Solka-Floc, Brown Company, Berlin, New Hampshire.

[6] Two per cent cellulose used in the soybean protein basal diet, when the diet was compared to the dried skim milk diet, to reduce the iron content of the diet.

in experiments 1 and 2 was treated twice with Na_2EDTA while the soybean protein used in experiment 3 was treated 4 times.

After the treatments, the protein was suspended in distilled water, heated, stirred, and allowed to settle. The washing with distilled water was repeated 5 times. In order to test if all of the Na_2EDTA had been removed by washing, 10 ml of the last supernatant was added to 5 ml of a saturated solution of ammonium oxalate, and the pH adjusted to pH 11.0 with sodium hydroxide. On addition of one drop of saturated solution of calcium chloride the presence of a precipitate showed that the EDTA had been removed. Finally, as much water as possible was removed by a hydraulic press and the protein was dried in an oven at 50°C.

The untreated and treated proteins and the other nonmineral ingredients of the basal diets were analyzed for calcium, magnesium, phosphorous, zinc, manganese, copper, iron and molybdenum by the x-ray fluorescent spectrometer and the chemical methods of Johnson and Ulrich ('59). The results are presented in table 2. The amount of iron and copper in these ingredients, and the amount of these minerals supplied by the reagent grade chemicals in the mineral mixture were used to calculate the iron and copper content of the basal diets. The results of the calculation are presented in table 3.

Blood samples were obtained by cardiac puncture and transferred immediately to heparinized tubes. The hemoglobin content of the blood was determined by the cyanmethemoglobin method in experiments 1 and 2. The modified alkaline-acid hematin method of Bankowski ('42) was used in experiment 3. The values for packed cell volume (PCV) were obtained with a microcapillary centrifuge.

The results presented in this report were subjected to statistical analysis according to procedures described by Snedecor ('56).

RESULTS AND DISCUSSION

Effect of protein treatment. Washing the soybean protein with water was nearly as effective in removing calcium and magnesium, as was washing the protein with Na_2EDTA, solution (table 2). The Na_2-EDTA, however, was more effective in removing manganese, copper, iron, molybdenum and zinc, particularly when the number of treatments was increased. Iron, and to some extent copper, were so tightly bound to the protein that additional treatments and increasing concentrations of Na_2EDTA did not remove as much of these elements as of manganese, molybdenum and zinc. The phosphorus content of the protein was not altered by the treatment, because EDTA is a negatively charged anion and will not chelate with other anions.

Iron. In experiment 1, the basal diet with the untreated soybean protein contained 45.3 mg of Fe and 10.5 mg of Cu/kg of diet, while the basal diet with the treated protein contained 36.3 mg of Fe and 8.0 mg of Cu/kg of diet. The amounts of copper in these diets included

TABLE 2

Average trace mineral content of untreated and treated soybean protein and of the other nonmineral ingredients used in the basal diets, dry-matter basis

	Moisture	Ca	Mg	P	Mn	Cu	Fe	Mo	Zn
	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Untreated soybean protein, avg A and B[1]	8.8	296	442	8,530	16	43	61	1.0	51
Water-treated soybean protein, A	6.1	35	28	8,763	4	50	68	0.6	32
Na_2EDTA-treated soybean protein, 0.5%, 2 times, avg A and B	6.8	24	13	8,400	6	23	29	0.3	2
Na_2EDTA-treated soybean protein, 0.5%, 3 times, A	6.7	48	19	8,850	3	16	29	0.4	0
Na_2EDTA-treated soybean protein, 0.5%, 4 times, A	8.3	29	15	8,837	4	7	43	0.3	2
Na_2EDTA-treated soybean protein, 1.0%, 3 times, A	7.0	65	12	9,490	1	16	26	0.3	1
Na_5ETPA[2]-treated soybean protein, 1.3%, 4 times, A	6.6	37	28	7,915	1	20	31	0.1	0
Untreated soybean protein, C[3]	6.1	—	—	8,548	8	24	117	7.4	31
Na_2EDTA-treated soybean protein, 0.5%, 2 times, C	7.5	43	213	8,417	3	14	81	4.0	1
Na_2EDTA-treated soybean protein, 0.5%, 4 times, D[4]	9.0	146	28	8,854	9	9	48	0	5
Dried skim milk	12.9	9,631	308	6,817	2	6	28	0	40
Cornstarch	12.5	369	73	448	6	3	12	0	0
Cellulose	0.2	190	108	380	9	4	172	0	4
Soybean oil	0.3	70	4	380	0	1	20	0	0
Chick starter mash	10.8	15,700	1,790	10,550	268	16	115	9.8	107

[1] Samples A, B and C and D are different lots of isolated soybean protein.
[2] Versenex-80, Dow Chemical Company, Midland, Michigan.
[3] Sample C was used in experiments 1 and 2.
[4] Sample D was used in experiment 3.

2.25 mg/kg furnished by the mineral mixture. The results of experiment 1 are given in table 4. The results show that the total iron, 45.3 mg/kg, in the untreated soybean protein supported approximately optimum growth. The hemoglobin level and the cell volume, however, were somewhat below the normal range. On feeding the treated protein, growth, hemoglobin level and cell volume were found to be significantly less than these observations on the chicks receiving the untreated protein basal diet ($P = 0.001$).[2]

The addition of iron to the treated protein basal diet increased chick growth, hemoglobin content and cell volume ($P < 0.005$). When 10 mg of Fe/kg were added to the treated protein diet, the total amount of iron supplied was 46.3 mg/kg, an amount equivalent to the unsupplemented, untreated protein diet. The hemoglobin and cell volume of the group receiving the 10 mg/kg of added iron, however, were considerably below the untreated protein basal. Only when 25 mg of Fe/kg were added, a total amount of 61.3 mg of Fe/kg of diet, were the blood levels increased to levels comparable to those of the chicks fed the untreated protein diets. It appears, therefore, that the iron added to the treated protein diet was not as available for growth, hemoglobin or cell volume as the iron from the untreated soybean protein.

The addition of Na_2EDTA to the treated protein basal diet decreased chick growth but had no effect when the diet was supplemented with iron ($P < 0.01$). Hemoglobin content and cell volume, however, were decreased significantly by Na_2EDTA, with and without addition of iron, to the treated protein basal diet ($P < 0.005$ and < 0.01, respectively). When 25 mg of

[2] Only one probability is given for the three criteria except when statistical analysis showed that they differed.

TABLE 3

Mineral content of basal diets

Experiment	Treatment	Cu	Fe
		mg/kg	*mg/kg*
1	Iron series		
	Untreated protein basal diet	10.5[1]	45.3
	Na_2EDTA-treated protein basal diet	8.0[1]	36.3
2	Copper and iron series		
	Untreated protein basal diet	8.2	45.3
	Na_2EDTA-treated protein basal diet	5.7	36.3
3	Treated soybean protein basal diet	9.6[2]	23.7
	Dried skim milk protein basal diet	11.1[1]	28.0

[1] Includes 2.25 mg added Cu/kg of diet.
[2] Includes 5 mg added Cu/kg of diet.

TABLE 4

Effect of Na_2EDTA on growth, hemoglobin content, packed cell volume and pigmentation of chicks fed soybean protein and Na_2EDTA treated soybean protein diets containing graded amounts of iron (exp. 1)

Treatment	Body weight	Hemoglobin	PCV[1]	Depigmentation score	Survivors/group
	gm	*gm/100 ml blood*	%		
Untreated protein basal diet	341	6.5	21.9	0.0	10/10
Treated protein	301	4.2	17.1	0.5	6/10
+ 10 mg Fe/kg	308	4.8	18.6	0.2	7/10
+ 25 mg Fe/kg	340	6.6	22.4	0.1	9/10
+ Na_2EDTA, 0.07%	269	3.1	13.2	0.9	6/10
+ Na_2EDTA + 10 mg Fe/kg	307	4.3	18.1	0.4	7/10
+ Na_2EDTA + 25 mg Fe/kg	353	5.5	20.0	0.1	9/10

[1] Indicates packed cell volume.

Fe/kg were added with the Na_2EDTA, a total of 61.3 mg/kg, the growth approached that of the group receiving the untreated protein diet containing 45.3 mg of Fe/kg, but the hemoglobin and cell volume were still below those of this group. Thus, the addition of Na_2EDTA to the treated protein diet made the iron remaining after the initial treatment of the protein less available for growth, hemoglobin and cell volume. Larsen et al. ('60) observed that 0.1% Na_2EDTA in the diet fed to rats decreased the absorption of radioactive ferric chloride and increased the excretion in the urine.

The anomaly, revealed in the results of experiment 1, is perhaps best explained by the probability that the tightly bound iron of treated isolated soybean protein is released when the protein is broken down into amino acids during digestion. Thus Na_2EDTA, although it combines with iron to form a complex with a high stability constant, is relatively ineffective in removing the bound iron from the protein but chelates readily with the iron digestion product. In turn Na_2EDTA renders iron less available for growth, hemoglobin formation and normal cell volume because the high stability constant of Fe-EDTA is equal to, or slightly greater than hemoglobin.

A high rate of mortality occurred in the iron-deficient groups, which decreased as the level of iron was raised. The mortality occurred suddenly between the second and third week of the experimental period.

In this experiment, the New Hampshire chicks receiving the iron-deficient diets became depigmented between the third and fourth week of the experimental period. The depigmentation continued and by the ninth week the chicks were almost white. Pigmentation was scored according to a scale in which values from zero to one indicated normal-to-completely depigmented feathers. Pictures of representative normal and depigmented 9-week chicks are presented in figure 1. The degree of depigmentation was increased by the addition of Na_2EDTA to the diet. The iron and copper content of the normal red feathers was 37.5 and 14.2 mg/kg, respectively, while the iron and copper content of the depigmented feathers was 25.5 and 10.0 mg/kg, respectively.

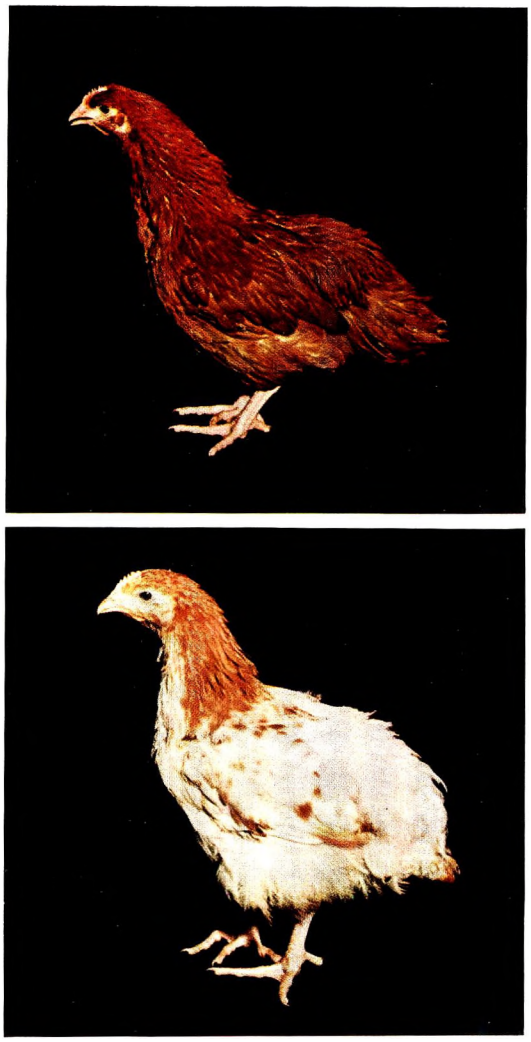

Fig. 1 Effect of an iron deficiency on feather pigmentation of New Hampshire chicks. Top chick fed isolated soybean protein diet. Bottom chick fed treated isolated soybean protein diet deficient in iron.

In order to obtain further information on the chick's requirement for iron, graded levels of iron and copper were fed in experiment 2. The basal diet with the untreated soybean protein contained 45.3 mg of Fe/kg, and 8.2 mg of Cu/kg of diet, while the basal diet with the treated protein contained 36.3 mg of Fe/kg, and 5.7 mg of Cu/kg of diet. No additional

TABLE 5

Effect of growth, hemoglobin content, packed cell volume, pigmentation and mortality of chicks fed Na_2EDTA treated soybean protein diets containing graded amounts of iron and copper (exp. 2)

Treatment	Body weight[1]	Hemoglobin	PCV[2]	Depigmentation score	Survivors/group
	gm	*gm/100 ml blood*	%		
Untreated protein basal diet	353	7.6	23.3	0.0	18/20
+80 mg Fe/kg+6.75 mg Cu/kg	374	9.0	25.6	0.0	19/20
Treated protein basal diet	220	5.0	19.0	0.6	8/20
No Fe+2.25 mg Cu/kg	257	4.7	16.9	0.6	9/20
No Fe+6.75 mg Cu/kg	256	5.0	18.5	0.8	9/20
+5 mg Fe+6.75 mg Cu/kg	288	5.1	19.5	0.5	11/20
+10 mg Fe+6.75 mg Cu/kg	273	5.2	18.8	0.5	15/20
+20 mg Fe+6.75 mg Cu/kg	303	5.6	19.5	0.2	15/20
+40 mg Fe+6.75 mg Cu/kg	307	7.2	22.2	0.1	13/20
+80 mg Fe+6.75 mg Cu/kg	366	8.2	23.9	0.1	20/20

[1] Average of duplicate groups.
[2] Indicates packed cell volume.

iron or copper was supplied by the mineral mixture used in the basal diets. The results of experiment 2 are given in table 5. A significant reduction was observed in growth, hemoglobin and cell volume of the chicks fed the treated soybean basal diet compared to the chicks fed the untreated soybean protein basal diet ($P < 0.005$).

A slight growth increase was obtained when 2.25 mg of Cu/kg was added to the treated soybean protein basal diet, making a total of 9.45 mg of Cu/kg, but an additional 4.5 mg of Cu/kg promoted no further response. Addition of copper failed, however, to affect either hemoglobin or cell volume. With the copper level held constant, chick growth in general was progressively increased with graded additions of iron. Addition of graded levels of iron also progressively increased hemoglobin level, and cell volume. The addition of 10 mg of Fe/kg, a total of 46.3 mg/kg, produced results which were markedly below the untreated protein diet, which contained a total of 45.3 mg of Fe/kg of diet. This confirmed the results obtained in experiment 1.

A high rate of mortality occurred in the iron-deficient groups in this experiment as in experiment 1. This was reduced as the iron level increased. Autopsy of the chicks from the iron-deficient groups showed that the kidneys were slightly enlarged and somewhat hemorrhagic. The spleens were smaller than normal and quite pale, which would be expected in anemic birds.

Depigmentation of the feathers due to iron deficiency was observed again in this experiment, and increased pigment deposition occurred as the level of iron was raised. The groups receiving the highest level of copper, but no iron, showed the greatest loss of feather pigment. This indicates that copper deficiency did not enhance the depigmentation caused by iron deficiency. Moreover in previous work,[3] no depigmentation of feathers was observed in chicks fed a low-copper diet adequate in iron.

Achromotrichia has generally been found to be associated with a copper deficiency in the rat, rabbit, cat, dog, sheep and cattle, (Lerner and Fitzpatrick, '50). Hill and Matrone ('61) observed a somewhat reduced intensity of the color of the feathers of Rhode Island Red chicks in iron deficiency, but the greatest change in pigment concentration occurred in chicks receiving a copper-deficient diet. Miller and Denton ('59) reported that chicks fed high levels of molybdenum and sodium thiosulfate had depigmented feathers and that copper added to the diet produced normal feather color. Rothman and Flesch ('43) found that human red hair, after it had been dehydrated, rendered

[3] Davis, P. N., L. C. Norris, F. H. Kratzer, 1962, unpublished data.

free of lipids, partially disintegrated and treated with dilute acid, contained about 400 mg/kg of a red, iron-containing pigment not encountered in other hair. Nickerson ('46) isolated from red feathers what appeared to be the same iron-containing red pigment as that which Rothman and Flesch ('43) had recovered from human red hair. Stout et al. ('60) reported that the "cotton-fur" abnormality in mink was not prevented with 11 parenterally administered B-vitamins, parenteral copper or oral lysine plus tyrosine, but that parenteral iron prevented loss of pigment from the fur.

The immediate cause of the achromotrichia observed in this investigation is unknown. Apparently, however, when a severe dietary iron deficiency exists, and the chick becomes depleted of its body reserves, the requirement of iron for hemoglobin formation, and other tissue needs is more critical than for feather pigmentation and deposition ceases, resulting in the growth of new feathers containing no pigment. When an adequate level of iron is again supplied the deficient chicks, deposition of pigments again takes place in the new feathers.

In addition to the need of iron in red feather pigments, the possibility also exists that iron functions in an enzyme system involved in this process, as copper in tyrosinase, and that the enzyme is prevented from functioning when iron is deficient. The strain of New Hampshire chicks used had feathers containing areas of black melanin along with the red pigment. In the depigmented feathers, neither the black nor the red pigment was deposited. When a diet containing iron was fed the deficient chicks, the new feathers contained first a band of black pigment followed by the red pigment. As the feathers grew, the black melanin was distributed throughout the feathers in the normal pattern.

In experiment 3, additional evidence on the availability of iron in soybean protein was obtained by comparing treated soybean protein and dried skim milk basal diets containing graded levels of iron. In this experiment hemoglobin was determined by the modified alkaline-acid hematin method of Bankowski ('42), rather than the cyanmethemoglobin method. The treated soybean protein basal diet contained 23.7 mg of Fe and 9.6 mg of Cu/kg and the dried skim milk basal diet contained 28.0 mg of Fe and 11.1 mg of Cu/kg. The basal diets contained 5 mg of added Cu/kg, but no added iron. The results of the experiment are given in table 6.

The results indicated that each addition of iron to either basal diet improved growth, hemoglobin and cell volume ($P < 0.005$). The hemoglobin and cell volume values of the chicks receiving the dried skim milk diets were higher than the comparable values of the chicks given the isolated protein diets ($P < 0.005$).

TABLE 6

Effect on growth, hemoglobin content, packed cell volume, iron consumption, and mortality of chicks fed Na_2EDTA-treated soybean protein and dried skim milk diets containing graded amounts of iron (exp. 3)

Treatment	Body weight[1]	Hemoglobin	PCV[1]	Fe consumed/ 100 gm gain	Survivors/ group
	gm	*gm/100 ml blood*	*%*	*mg*	
Treated soybean protein basal diet	213	3.6	15.0	8.6	5/10
+ 40 mg Fe/kg	435	8.9	23.6	12.2	8/10
+ 80 mg Fe/kg	462	10.3	26.6	18.9	10/10
+ 160 mg Fe/kg	426	10.0	26.2	33.1	10/10
Dried skim milk basal diet	210	4.4	17.6	12.7	5/10
+ 40 mg Fe/kg	422	10.7	27.7	18.8	7/8
+ 80 mg Fe/kg	436	11.1	27.5	26.2	9/9
Practical chick starter diet	469	10.4	26.0	—	10/10

[1] Indicates packed cell volume.

The amount of diet consumed by the groups receiving the dried skim milk diets, however, was greater than that consumed by treated soybean protein groups. On the average, the groups fed the skim milk diets consumed 6.0 mg of Fe/100 gm of gain more than the comparable groups receiving the treated soybean protein diets. This probably accounts for the blood of the chicks consuming the dried skim milk diets containing a higher level of hemoglobin and greater cell volume. Apparently, therefore, the iron in isolated soybean protein is approximately as available as the iron in dried skim milk.

The National Research Council ('60) recommends a level of 20 mg of Fe and 2 mg of Cu/kg of diet. On the other hand, Hill and Matrone ('61) suggest, on the basis of blood data alone, that a diet containing 40 mg of added Fe and 4 mg of added Cu/kg would be closer to the chicks' requirement. Both of these recommendations appear low if growth, hemoglobin, cell volume and mortality data are used as criteria. It appears that the chicks' requirement is between 65 and 105 mg of total iron/kg of diet containing approximately 10 mg of total copper/kg when either treated isolated soybean protein or dried skim milk is used as the protein source.

SUMMARY

In experiments on freeing isolated soybean protein of its mineral elements, the levels of zinc, molybdenum, manganese, calcium and magnesium were reduced approximately 35, 40, 75, 85 and 95%, respectively, by washing the protein with water. When the protein was treated with the disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) or the pentasodium diethylenetriaminepentoacetic acid (Na_5ETPA), the levels of these mineral elements were decreased 100, 70, 80, 85 and 95%, respectively. Copper and iron were not reduced by the water treatment, but were lowered approximately 65 and 50%, respectively, by the Na_2EDTA treatment.

In studies with chicks, fed diets containing Na_2EDTA-treated isolated soybean protein, together with graded amounts of iron, the iron from the untreated soybean protein was found to be available for growth, hemoglobin formation and cell volume. On the other hand, iron which was added to the treated soybean protein basal diet was not as available for growth, hemoglobin formation or cell volume as the iron from the untreated soybean protein basal diet. The iron remaining in the protein after the Na_2EDTA treatment and the graded levels of iron added to the treated soybean protein basal diet were less available for growth, hemoglobin and cell volume, when Na_2EDTA was added to the diet than when the diet contained no added Na_2EDTA. Iron deficiency caused depigmentation of the feathers of New Hampshire chicks which was not related to the copper level of the diet. The results of the investigation showed that, under some conditions, the iron requirement of the chick is greater than that previously reported.

ACKNOWLEDGMENTS

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Gamma Irradiation and Interrelation of Dietary Vitamin A and Copper on Their Deposition in the Liver of Swine[1,2]

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Copper and vitamin A have been shown to be interrelated in a number of dietary studies. Halverson and Hendricks ('55) reported that copper caused a loss of vitamin A in stored poultry diets. The addition of 0.2 ppm of copper to a peanut oil solution of vitamin A destroyed its activity (Bhattacharya et al., '54). Other workers (Kamstra et al., '52) found that traces of copper decreased storage stability of carotene in carrot oil. Balakhovskii et al. ('52) observed that carotene protected ascorbic acid from oxidation by copper, and Balakhovskii and Drozdova ('57) discussed keratinization of tissues in the absence of vitamin A as due to oxidative catalysis by copper. These references report not only that copper oxidizes vitamin A in a variety of materials, but that carotene and vitamin A protect other substances in feedstuffs and metabolizable tissues from oxidation by copper catalysis.

The present study was made to determine (1) whether dietary vitamin A had an effect on the level of deposition of copper in the liver; (2) whether dietary copper had an effect on the deposition of vitamin A in the liver; and (3) whether whole body gamma irradiation had any effect on the deposition of vitamin A and copper in the liver of swine.

EXPERIMENTAL

Two experiments were conducted. In the first experiment, 28 pigs, two weeks of age, were randomly divided into two dietary groups; one group received the basal ration (table 1) that contained carotenoids equivalent to 150 IU of vitamin A per pound of feed; for the other group 2,000 IU of vitamin A as the acetate were added per pound of feed. After the pigs were fed the diets for 13 weeks, 7 pigs of each dietary group were irradiated with 350 roentgens (r) of gamma rays over the whole body by a 6,000-curie Co^{60} source. The dose was given at the rate of 11.1 r/min. The irradiated pigs survived an average of 11 days after dosage. The non-irradiated pigs were killed after being fed the diets for approximately 16 weeks.

TABLE 1

Composition of rations

	Experiment 1[1]	Experiment 2
Ground white corn	58.2	—
Ground yellow corn	—	80.35
Soybean meal, 50% protein	23.6	17.0
Cane sugar	10.0	—
Stabilized fat	3.0	—
Steamed bonemeal	2.0	1.0
Salt	0.5	0.5
Ground limestone	—	1.0
Trace minerals (Mn, Fe, Cu, Co, Zn, I)	0.1	0.05[2]
Vitamin B supplement[3]	0.3	—
Vitamin D and E supplement[4]	2.0	—
Vitamin premix[5]	—	0.10

[1] Ration contained 150 IU of vitamin A in the equivalent form of carotenoids. When vitamin A acetate was added it was added at the level of 2,000 IU of vitamin A/pound of feed.

[2] Contributed 22 ppm of copper to basal ration, by analysis.

[3] Supplied in millgrams per pound of feed: riboflavin, 8; pantothenic acid, 8; choline chloride, 20,000.

[4] Supplied in IU/pound of feed: vitamin D, 400; vitamin E, 25.

[5] At this level each 100 pounds of feed contained: vitamin A, 100,000 IU; vitamin D, 9,000 IU; vitamin B_{12}, 450 mg; niacin, 900 mg; riboflavin, 200 mg; pantothenic acid, 400 mg; choline chloride, 980 mg; and 0.02 pounds of calcium.

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In experiment 2, 40 weanling pigs were fed a basal ration with the composition shown in table 1. These pigs were divided into 4 dietary groups as follows: (1) basal; (2) basal plus 150 ppm of copper; (3) basal plus 150 ppm of copper for 6 weeks and then 75 ppm; and (4) basal plus 150 ppm of copper for 6 weeks and then the unsupplemented basal diet until slaughtered. The copper was added as copper sulfate. The swine were slaughtered at approximately 190 pounds of weight, and samples of liver were obtained and frozen immediately at − 8°C and analyzed within a few days for vitamin A and copper.

Vitamin A was determined by the antimony trichloride-acetic anhydride procedure of Gallup and Hoefer ('46). Copper was determined by the carbamate-versenate method of Cheng and Bray ('53). Statistical analysis of the data for the significance of the variance due to the treatments was done according to Snedecor ('56).

RESULTS AND DISCUSSION

In figure 1, data are presented for the concentration of vitamin A in the liver of the pigs of the first experiment. The vitamin A unsupplemented dietary groups had only 2 to 3 IU of vitamin A/gm of fresh liver. The high vitamin A values (116 to 122 IU/gm of fresh weight) in the livers of the supplemented groups demonstrate the remarkable capacity of the liver to store vitamin A when it is present in the diet. Heart and gracilis muscle of both dietary groups were also analyzed and were found to have the same level of vitamin A present as the liver of the pigs with no supplemental vitamin A. Irradiation with 630 r of gamma rays over the whole body had no significant effect on the deposition of vitamin A in the tissues.

Figure 1 also presents the data obtained in experiment 1 for the effect of dietary vitamin A, with and without irradiation, on the concentration of copper in the liver. The swine that received no supplemental vitamin A had a greater concentration of copper in the liver ($P < 0.05$). The data were calculated on the basis of the amount of copper present in the ash of the liver and the same effect of the treatments existed. Irradiation resulted in a greater deposition of copper in the liver ($P < 0.01$).

The alimentary membranes are markedly affected by irradiation, and this may have aided greater absorption of copper

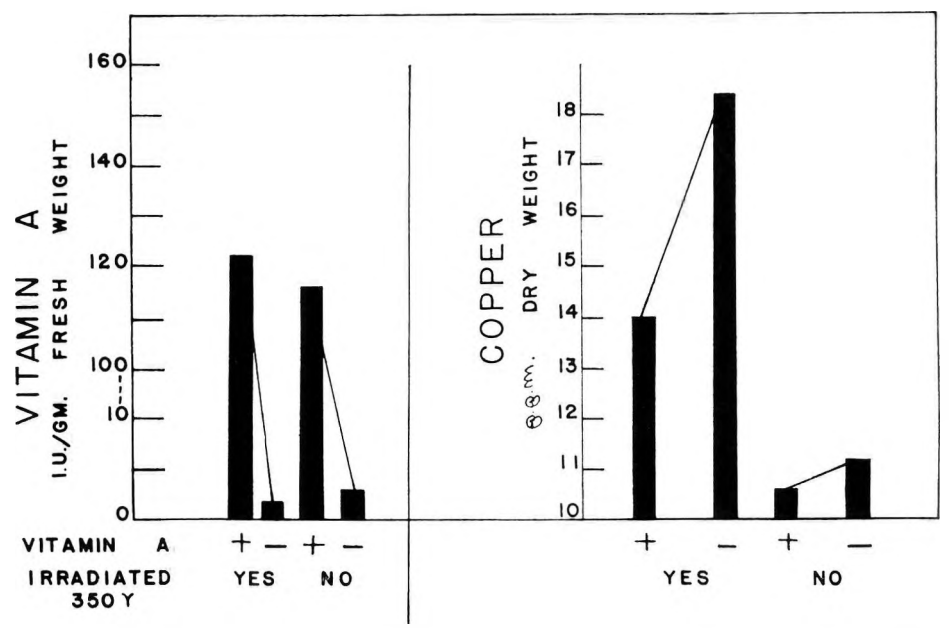

Fig. 1 Effect of dietary vitamin A and gamma irradiation on vitamin A and copper concentration in the liver of swine.

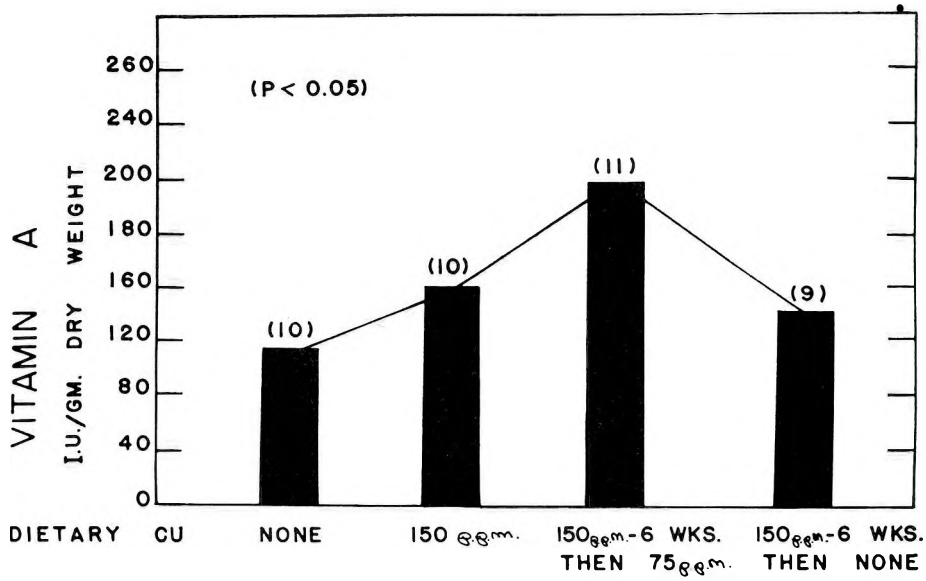

Fig. 2 Effect of dietary copper on the deposition of vitamin A in the liver of swine.

from the tract and thereby caused the increase in level of copper in the liver of the irradiated animals. However, dietary vitamin A and the gamma irradiation did not effect deposition of copper in the heart and gracilis muscle.

Figure 2 presents the data obtained in the second experiment for the copper on the concentration of vitamin A in the liver, expressed as international units per gram of dry weight. The copper treatments resulted in an increase ($P < 0.05$) in the amount of vitamin A in the liver. This relationship also held ($P < 0.05$) when the international units of vitamin A were calculated per gram of fresh weight. The increased vitamin A in the liver may have been due to the copper sulfate's giving better intestinal health through parasite control and thereby increasing intestinal absorption of the vitamin A.

SUMMARY

A study was made to determine (1) whether dietary vitamin A had an effect on the level of copper deposition in the liver; (2) whether dietary copper had an effect on the deposition of vitamin A in the liver; and (3) whether whole body gamma irradiation had any effect on the deposition of vitamin A and copper in the liver of swine.

Vitamin A supplementation decreased the deposition of copper in the liver.

Copper supplementation increased the level of vitamin A in the liver.

Gamma irradiation increased the deposition of copper in the liver.

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Performance of Rats Fed Fish Flour or Casein as the Sole Source of Dietary Protein Through Four Generations

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Studies in this laboratory with rats (Metta, '60)[2] have demonstrated that a fish flour which has been desiccated, defatted and deodorized by azeotropic solvent extraction with ethylene dichloride promotes growth equivalent to 91% of that from whole egg protein using the paired feeding method for 21 days (Mitchell and Beadles, '30); has a better protein efficiency ratio[3] (PER) than skim milk or beef protein and 90% as great as that of whole egg protein; has a biological value (Mitchell, '24) which is comparable to milk and better than that of beef protein; and has significantly increased the PER and mineral content of 4 representative Indian vegetable diets as well as the PER of low-protein and high-protein corn diets when fed to rats at a concentration (3%) which was not detected by a panel of 26 Indian subjects.

The purpose of the present paper is to report data concerning the acceptability of fish flour by rats when fed as the sole source of dietary protein in diets otherwise nutritionally adequate; to test for evidence of acute or chronic toxicity over several generations; and to make other observations on the general performance of these rats.

EXPERIMENTAL

The fish flour used in these trials was taken from a batch prepared on plant scale (300 pounds) in February, 1957.[4] The whole fish were desiccated, defatted and deodorized by azeotropic solvent extraction with ethylene dichloride (Levin and Finn, '55). The flour had been stored at room temperature for 4 years prior to use in these studies, and proximate analysis at the time of usage showed that it was composed of: 79.1% crude protein (N × 6.25), 15.1% ash, 1.1% ether extractable substances, 95.6% dry matter, and had 4.33 Cal. gross energy/gm. The experimental diets (table 1) contained 18.8% of crude protein either as fish flour or casein supplemented with methionine. Food and water were provided ad libitum.

TABLE 1
Composition of the 19% protein diet

	%	%
Fish flour[1]	24.8	
Casein[2]		22.3
Methionine		0.2
Starch	49.2	47.0
Sucrose	10.0	10.0
Corn oil	5.0	5.0
Wheat-germ oil	3.0	3.0
Vitamin mixture[3]	5.0	5.0
Mineral mixture[3]	1.0	5.5
Cellulose[4]	2.0	2.0

[1] Prepared by azeotropic solvent extraction of whole fish using ethylene dichloride on plant scale by Vio Bin Corporation, Monticello, Illinois. The fish flour was generously supplied through the courtesy of Dr. Ezra Levin.
[2] Labco Vitamin-Free Casein, The Borden Company, New York.
[3] As used by Schendel and Johnson ('54) plus 10 mg of thiamine·HCl and 50 mg of Ca pantothenate/kg diet. Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, cyanocobalamin, pteroylglutamic acid, menadione, biotin, α-tocopheryl succinate and *p*-aminobenzoic acid were generously supplied by Merck Sharp and Dohme, Rahway, New Jersey, through the courtesy of Drs. Laurent Michaud and S. F. Scheidy. Methionine was generously supplied by Dow Chemical Company, Midland, Michigan, through the courtesy of Dr. Julius Johnson. Water-soluble vitamin A and vitamin D were generously supplied by Endo Products Company, Richmond Hill, New York, through the courtesy of Dr. S. M. Gordon.
[4] Woodflock, Brown Company, Berlin, New Hampshire.

The design for these studies on reproduction was to mate all female rats at 100

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[2] Metta, V. C., and S. Metta, unpublished data.
[3] PER = grams gain in body weight/gram protein consumed.
[4] Vio Bin Corporation, Monticello, Illinois.

days of age and wean the young at 21 days of age. The females were remated again 10 days after the weaning or death of their litter. The original breeding stock was composed of weanling rats of the Sprague-Dawley strain: 15 male and 15 female rats received the fish flour diet, and 5 male and 5 female rats received the casein diet. The original males used in the first two matings were maintained with their respective diets until killed and examined histologically. Succeeding matings were conducted with weanling males obtained from a commercial source (Sprague-Dawley) and raised to maturity (100 days of age) with commercial rat chow. These breeding males were replaced every second mating. The male pups resulting from any of the matings were fed their respective diets until they were killed, and examined histologically. The number of pups born in each litter was not reduced to a common number, but all surviving offspring were carried to weaning. Such a plan provided information concerning the viability of the young but prevented the birth and weaning weights from being comparable.

Histological data[5] were accumulated at two times during the course of the experiment. Approximately midway through the study (after 18 months), 84 animals of the first three generations were killed and examined for evidence of pathology. Forty additional rats were killed at the end of the study (after 12 months). These were progeny from the second mating of the second generation females.

The rats were anesthetized and exsanguinated via the carotid arteries and jugular veins. The following organs and tissues were blotted on filter paper, weighed and then preserved in a 10% formalin solution made up in 0.9% saline: liver, kidney, spleen, heart, lung, stomach, duodenum, adrenal glands, thyroid glands, testes, epididymus, ovaries, oviducts, uterus and gastrocnemius muscle. The identification of these specimens was not disclosed to the histologists until after the gross and microscopic examinations were completed.

RESULTS

The reproductive performance of rats receiving fish flour or casein as their sole source of dietary protein is compared and summarized in table 2. Data on the first generation were accumulated from three matings of the parent animals. Data on the second generation were accumulated from 7 consecutive matings of the female offspring. The progeny from the first of these matings were then mated 7 times and provide the data on the third generation. Additional data on the fertility of rats consuming the fish flour diet were accumu-

[5] The authors are indebted to Professor A. C. Ivy of the Department of Clinical Science, University of Illinois, Chicago, and Dr. S. Bernick of the Department of Anatomy, University of Southern California, Los Angeles, for the histological examination of the various tissues.

TABLE 2

Reproductive performance of rats fed fish flour or casein as the sole dietary protein through three generations

Generation	Pregnancy rate		Litter size		Weaning rate	
	Casein[1]	Fish flour[1]	Casein[1]	Fish flour[1]	Casein[1]	Fish flour[1]
	% of matings		*no. born/litter*		*% of pups born*	
F_1, 3 matings	9/12	35/59	98/9	302/31	33/98	119/254
	75%	56%	11 pups	10 pups	34%	47%
F_2, 7 matings	9/25	14/22	58/7	131/14	9/31	24/83
	36%	64%	7 pups	9 pups	29%	29%
F_3, 7 matings	3/7	14/21	16/2	142/14	2/16	32/142
	43%	67%	8 pups	10 pups	13%	32%
Total[2]	21/44	63/102	172/18	575/59	44/145	175/479
	48%	62%	9 pups	10 pups	30%	37%

[1] Sole source of dietary protein.
[2] In addition, 4 matings of the third generation and 5 matings of the fourth generation female rats consuming the fish flour diet, yielded 5/7 or 71% and 16/22 or 73% pregnancies, respectively.

lated through 4 matings of the third generation and 5 matings of the fourth generation female rats.

The data indicate that rats consuming the fish flour diet were able to perform as well as the rats consuming the casein diet. In fact there is some suggestion that the rats consuming the fish flour diet were better able to withstand the exposure to the stress imposed in these studies than the rats consuming the casein diet. At no time did we observe any indication of toxicity in rats consuming the fish flour: neither from the reproductive performance of the animals nor from the wet weight or the gross and microscopic examination of 12 tissues and organs.

DISCUSSION

We have shown (Metta, '60) that the nutritive value, i.e., biological value (nitrogen balance) and protein efficiency ratio (growth test) of the fish flour protein compared very favorably with such high-quality proteins as whole egg, casein and beef and suggested that therefore it might be a valuable protein supplement. Under the conditions of those studies, however, the nutritional potential of the proteins might not have been fully demonstrated or measured. The deficiencies or limitations of a particular protein might only become manifest under the more rigorous conditions of a long-term study similar to the one described in this report where the test protein is the sole source of dietary protein and where some stress has been involved.

If one uses the reproductive performance of the rat consuming the casein diet as a standard, it is clear that these studies were conducted under conditions of stress. Although the animals used were purchused as wealings and maintained in a temperature- and humidity-controlled environment throughout the trial, they began to show signs of a respiratory involvement after several months, i.e., heavy breathing, sneezing and coughing. The experimental design might also be partly responsible for the poor reproductive performance of the animals, i.e., frequent mating, no reduction in litter size, and other variables. However, it is reasonable to assume that all animals were more or less equally challenged by the conditions of the study.

Under these conditions the reproductive performance of animals consuming the casein diet deteriorated appreciably over three successive generations. Since the pups appeared normal at birth and then became progressively emaciated, it appeared that this deterioration was the result of lactation failure. The dams also became emaciated during lactation. In the case of the rats consuming the fish flour diet, however, the effects of stress on reproductive performance were not nearly as marked. The females appeared in better health and achieved somewhat better pregnancy and weaning rates.

Although Miller ('56) has shown that methionine is the most limiting amino acid for rats fed heat-damaged fish meal, his data do not indicate whether such a limitation is a function of heat treatment or the amino acid composition of the fish meal. Recent reports by Njaa ('61) and Smith and Scott ('62), however, indicate that the sulfur-bearing amino acids are the most limiting for chicks fed fish meal that has not had its nutritional quality impaired by heat treatment. In the studies reported here we noted a sparseness in hair coat of pups at the time of weaning, in 4 out of 59 litters (7%) born to female rats fed the fish flour diet. These data might suggest that the most limiting amino acids of a high quality fish flour for rats are the sulfur-bearing amino acids.

SUMMARY

When fed as the sole source of dietary protein, a desiccated, defatted and deodorized fish flour prepared from whole fish by azeotropic solvent extraction using ethylene dichloride proved to be superior to casein for the reproduction and general performance of rats through three matings of the first generation, 7 matings of the second generation and 7 matings of the third generation. The pregnancy rate of animals consuming the fish flour was maintained through an additional 4 matings of the third generation and 5 matings of the fourth generation. Histological examination of 12 organs and tissues from animals of the first, second and third generations showed no differences between animals receiving fish flour or casein protein.

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Effects of Vitamin K-Active Compounds and Intestinal Microorganisms in Vitamin K-Deficient Germfree Rats

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In 1948 Gustafsson reported deaths of germfree weanling rats with symptoms suggestive of vitamin K deficiency. Luckey and co-workers ('55), however, were unable to demonstrate prolongation of blood clotting time in the Lobund strain of germfree rats maintained with a diet low in vitamin K. Gustafsson ('59b) reported the development of clear-cut vitamin K deficiency symptoms in a Swedish strain of germfree rats receiving a diet deficient in vitamin K. In a recent communication Brambel[4] reported the successful production of vitamin K deficiency symptoms in the Lobund germfree rat when the diets were rigorously extracted to remove traces of vitamin K. In Gustafsson's ('59b) experiments removal of the vitamin K-deficient animals from the germfree environment resulted in a return to normal prothrombin values. The rate at which this change occurred appeared to be related to the degree of "contamination" of the animals. Ex-germfree rats, moved to a conventional animal room, showed normal prothrombin times within 48 hours, whereas those kept in sanitized, but not germfree, containers did not fully revert to normal values even after 72 hours. These results suggested that members of the microflora of the conventional animals were involved in the recovery phenomenon. Consequently one objective of the present investigation was to determine whether single microorganisms could substitute for a complex microflora in providing vitamin K-active substances to the vitamin K-deficient germfree rat and thus to provide information on the role of specific members of the intestinal flora in the synthesis of vitamin K.

In a preliminary study, Gustafsson ('59b) administered various vitamin K-active compounds to germfree vitamin K-deficient rats by stomach tube and found that vitamin K_1 at a level of 1 mg/kg of body weight, (the only level tested) caused a return to normal prothrombin values within 8 hours. Comparable doses of menadiol tetrasodium diphosphate were not fully effective even at 16 hours. Menadiol sodium sulfate or 2,3-methyl-1,4-naphthohydroquinone did not increase the prothrombin values of the rats above 10% at 16 hours. As noted at the time, the animals were removed from the germfree isolators to sanitized containers to facilitate the taking of repeated blood samples; consequently some possibility existed of uncertainty that the observed results were due solely to the drugs administered. In the present study we therefore investigated the effects of various vitamin K-active compounds in vitamin K-deficient germfree rats maintained continuously in a germfree environment for the duration of the experiment. In addition to the peanut oil-containing diet used in the previous study (Gustafsson, '59b), the effect of two modifications of this diet were also studied, namely, a fat-free diet and a diet high in lard content.

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METHODS

Weanling rats of the Lobund germfree strain were studied at the National Institutes of Health. Other experiments were performed in the germfree colony at the University of Lund, Sweden (Gustafsson, '48, '59a). In both studies isolators of the Gustafsson type ('48, '59a) were used. The composition of the basal, fat-free diet is shown in table 1. Variations of this diet included the substitution of 10% of peanut oil or 25% of stripped lard for the corresponding amount of potato starch. The casein and the starch were previously extracted with petroleum ether for 24 hours. All diets were sterilized by autoclaving for 20 minutes at 121°C.

TABLE 1

Composition of basal diet

	gm
Casein, "vitamin-free" [1]	220
Potato starch[2]	720
Salt mixture[3]	40
Vitamin mixture[4,5]	+

[1] "Vitamin Free" Casein, General Biochemicals, Inc., Chagrin Falls, Ohio; extracted 24 hours with petroleum ether.

[2] Extracted 24 hours with petroleum ether.

[3] HMW, General Biochemicals, Inc. (Hubbel et al., '37).

[4] Water-soluble vitamin solution 661 used in the vitamin K-deficient diets contained the following for each 10 kg of diet: (in grams) thiamine·HCl, 0.5; riboflavin, 0.2; pyridoxine·HCl, 0.2; Ca-D-pantothenate, 1.0; nicotinamide, 2.0; *i*-inositol, 10.0; p-aminobenzoic acid, 3.0; biotin, 0.01; folic acid, 0.2; vitamin B_{12}, 0.0002; L-ascorbic acid, 10.0; and choline chloride, 20.0. These vitamins were dissolved in 40% ethanol and made to a volume of 200 cm^3; 20 cm^3 of solution used per kilogram of diet.

[5] Fat-soluble vitamin solution 664 used in the vitamin K-deficient diets contained the following for each 10 kg of diet: (in grams) vitamin A acetate, 0.07; calciferol, 0.001; and α-tocopherol, 5.0. These vitamins were dissolved in ethanol and made to a volume of 100 cm^3; 10 cm^3 used per kilogram of diet.

Control animals of the same strains as the germfree animals were housed in the animal room in cages with raised screen bottoms and given the same autoclaved diets as the germfree animals.

Two main types of experiments were performed. In the first, a terminal experiment, the animals were untreated, time of death was recorded and autopsies with histological examination were performed. In the second type of experiment when signs of severe hypoprothrombinemia were present, the animals were subjected to tests of the curative effect of menadione derivatives or monoinfection with specific microorganisms.

Prothrombin levels were determined according to a slightly modified Quick method (Lehman, '41). Prothrombin activity was calculated from a standard curve which was compiled for each lot of thromboplastin. With the thromboplastin preparations used, prothrombin activity levels of 10% corresponded to prothrombin clotting times of 50 to 60 seconds.

RESULTS

Development of vitamin K deficiency. With the basal diet all 10 germfree Lobund rats developed severe vitamin K deficiency with extensive hemorrhages and 100% mortality, as did all 17 germfree rats of the Swedish strain. The mean survival times on a sex basis were almost identical; that is, 28 and 33 days for the males and 44 and 46 days for the females of the Lobund and Swedish strains, respectively. The relationship between sex and susceptibility to vitamin K deficiency becomes more apparent when the data for the combined strains are plotted in the form of survival curves as shown in figure 1.

Effects of dietary fats. The effects of dietary fats on the survival time of vitamin K-deficient germfree rats are summarized in table 2 in which the data for

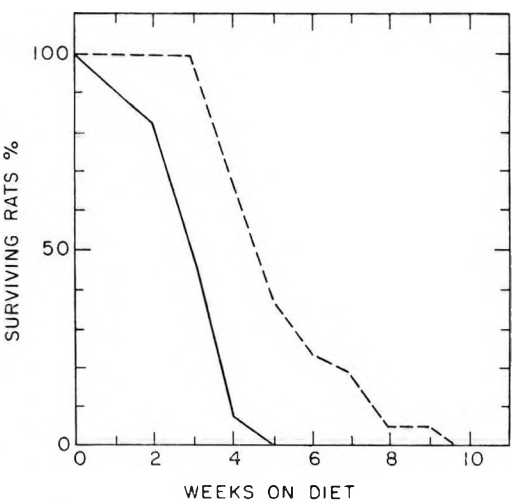

Fig. 1 Survival rate of 12 male (solid line) and 15 female (broken line) germfree rats fed the vitamin K-deficient basal diet.

TABLE 2

Effect of fats on survival of rats fed vitamin K-deficient diets

Bacteriological status	Diet	Sex	No. of rats	Mortality	Days fed diet until dead	
					Mean	Range
				%		
Germfree	Basal	male	12	100	30	12–35
		female	15	100	45	26–69
	Basal + lard, 25%	male	2	100	17	13–21
		female	8	100	16	9–26
	Basal + peanut oil, 10%	male	9	100	52	37–75
		female	8	0[1]	—	—
Conventional	Basal		10	0[1]	—	—
	Basal + lard, 25%	both	10	0[1]	—	—
	Basal + peanut oil, 10%		12	0[1]	—	—

[1] Surviving 120 days.

both strains have again been combined. It is clearly apparent that the basal mortality was increased by the incorporation of lard in the diet and decreased by the substitution of peanut oil. The latter effect is most striking in the case of the female rats fed peanut oil, which survived the full 120-day experimental period without any symptoms of vitamin K deficiency other than slightly lowered prothrombin values. Figure 2 shows the survival curves of the female germfree rats fed the various diets. None of the conventional control animals exhibited vitamin K deficiency symptoms with any of the diets.

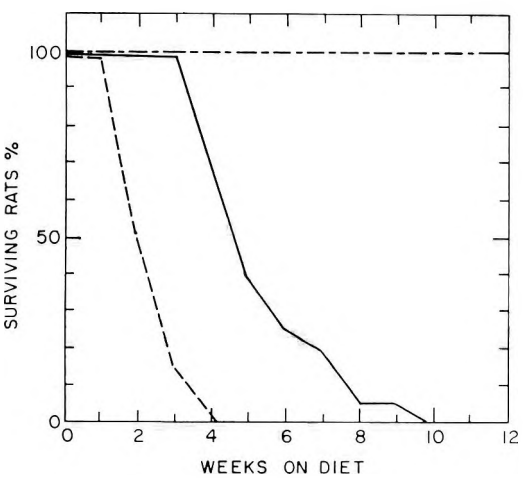

Fig. 2 Survival rate of female germfree rats fed the vitamin K-deficient basal diet (————); basal diet with 25% of lard (– – – – –); and basal diet with 10% of peanut oil (— — — — —).

Growth rate. It was suggested (Mameesh and Johnson, '60) that lack of vitamin K in the diet resulted in growth depression in conventional rats prevented from coprophagy by tail cups. In the present study weights of the animals were recorded only at death or the termination of the experiment. Nevertheless there were no grossly apparent differences in the size or weights of the experimental or control animals when comparisons could be made. For further clarification of this point three groups of 20 male weanling germfree rats of the Swedish strain were fed the basal diet, the basal diet containing 10% of peanut oil, or the basal diet containing 1.0 mg of vitamin K_1/100 gm. Some of the animals that succumbed to vitamin K deficiency showed slight growth depression during their final week when they experienced severe hemorrhages, but other animals gained between 20 and 30 gm during the same period. The growth curves of the animals are shown in figure 3. They were compiled from the mean weights of the surviving animals at each weekly weighing period. Even though the male rats were more severely affected than the females by the lack of vitamin K, and all the males eventually died except those given vitamin K_1, the rate of growth was satisfactory and almost equal with all three diets.

Reversal of vitamin K deficiency by vitamin K preparations. In previous studies (Gustafsson, '59b) it was found that prothrombin values of 10% or lower in germ-

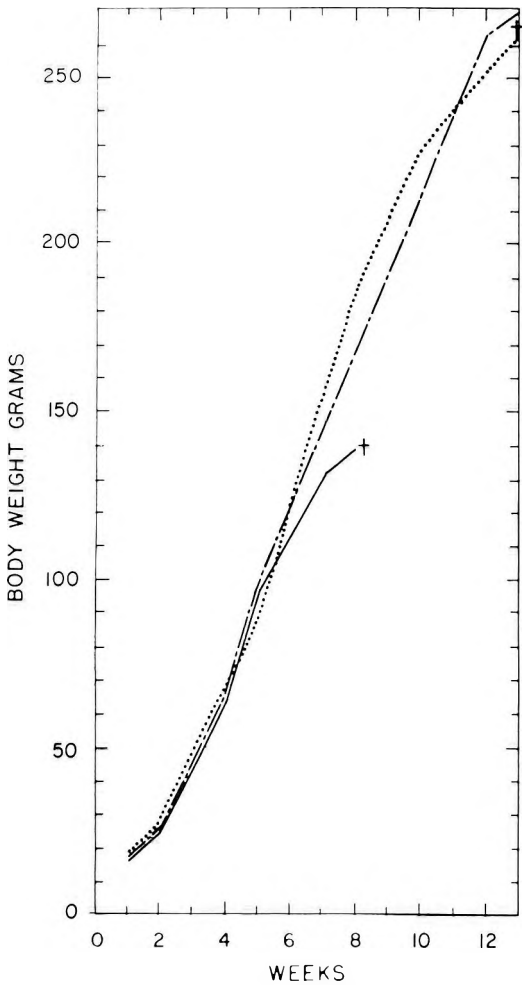

Fig. 3 Growth curves of germfree male rats fed the vitamin K-deficient basal diet and the basal diet supplemented with peanut oil or vitamin K at a level of 1 mg/100 gm of diet.

free rats could be brought to normal within 4 hours by oral doses of 1 mg of vitamin K_1/kg of body weight, whereas menadiol sodium sulfate was without effect at doses up to 5 times this level. Vitamin K deficiency symptoms have also been encountered with refined diets in which this latter compound was the sole source of vitamin K (Gustafsson, '48). In the present study vitamin K_1 at a level of 25 μg/kg of body weight had a full curative effect. As shown in table 3, menadiol tetrasodium diphosphate and menadiol sodium sulfate were less effective than vitamin K_1 by factors of ten- and one hundred-fold, respectively, measured by their effects on the prothrombin levels of vitamin K-deficient germfree rats. All of the preparations used were sterilized by filtration.

TABLE 3

Effect of menadione derivatives on prothrombin activity of vitamin K-deficient germfree animals[1]

Compound		Prothrombin activity 24 hours after dosing[2]
	μg/kg body wt	*%*
Vitamin K_1 (water-soluble)	25	100
Na menadiol diphosphate	25	< 10
	250	100
Na menadiol sulfate	25	< 10
	250	< 10
	1000	< 10
	2500	100

[1] Initial prothrombin activity < 10%; minimum of 3 rats/dose.
[2] Expressed as % normal.

Effect of microbial flora on vitamin K requirements. It had been shown earlier (Gustafsson, '59b) that exposure of germfree vitamin K-deficient rats to the full bacterial flora from conventional rats resulted in a return to normal prothrombin values within 24 to 48 hours. In the present study, attempts were made to identify the microorganisms responsible for the intestinal synthesis of vitamin K. A variety of microorganisms originally isolated from the oral cavity or the feces of rats were therefore tested by infecting vitamin K-deficient germfree rats with combined or single pure strains of these bacteria. Twelve strains in all were used and included lactobacilli, streptococci, micrococci, diphtheroids, bacterioids, coliforms and *Proteus*. In these tests germfree animals fed the vitamin K-deficient diet and which prothrombin levels at or below the 10% level were transferred to a simple type of isolator (fig. 4a, b) suitable for maintaining a single germfree animal for periods up to 14 days. The inoculum cultures were centrifuged, washed and resuspended in sterile saline to the original volume, in order to minimize the possibility of carry-over of vitamin K-active material from the growth medium. The inoculum was usually introduced by spraying

approximately 0.1 ml of a 24 to 48-hour culture into the isolator by means of a sterile syringe which pierced the air filter covering the isolator. Control germfree vitamin K-deficient animals showed no response to the uninoculated growth medium alone.

In three different experiments germfree male rats fed the basal diet plus 10% of peanut oil, with a bleeding tendency and prothrombin levels below 10% were monocontaminated and only an *Escherichia coli* strain and an unclassified sarcina-like micrococcus proved to be able to reverse the vitamin K deficiency symptoms. Results of a typical experiment are shown in table 4. In two separate experiments the prothrombin values of micrococcus-infected animals returned to normal within 24 hours.

The sarcina-like organism which reversed the vitamin K deficiency symptoms was an aerobic gram-positive nonmotile coccus. In various culture media the cells were arranged in pairs, tetrads, clumps and packets. The latter arrangement was also seen frequently in intestinal contents of monoinfected animals. There was a wide range of size for individual cells from about 0.7 to 2.5 μ, often in the same microscopic field. Colonies on nutrient agar or blood agar were light tan in color, circular and slightly raised with an entire margin. The colony size was 3 to 4 mm. In broth cultures growth was most rapid near the surface; with time a ring formed at the surface. Growth was rapid between 25° and 37°C. Acid, but no gas, was produced from glucose, levulose, maltose, sucrose, and trehalose within 48 hours. Lactose and melezitose were fermented more slowly (3 to 5 days) and cellobiose and glycerol only weakly. Adonitol, arabinose, dulcitol, galactose, mannitol, man-

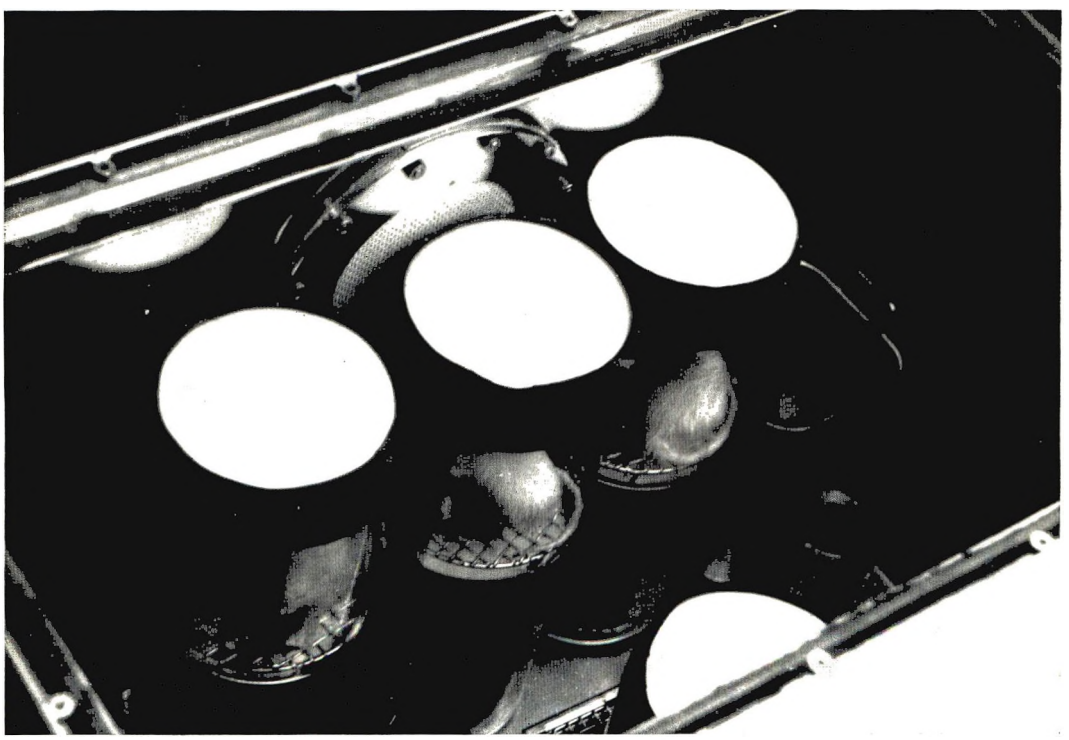

Fig. 4A Arrangement of 7 individual germfree isolators within the Gustafsson germfree apparatus prior to removal. The jars are covered with a glass fiber filter permitting access of air while preventing bacterial contamination. Each jar contains a supply of sterile food and water, in addition to a vitamin K-deficient rat. Inoculation of the animal is accomplished by introducing a saline suspension of the desired culture through the filter by means of a sterile syringe and needle.

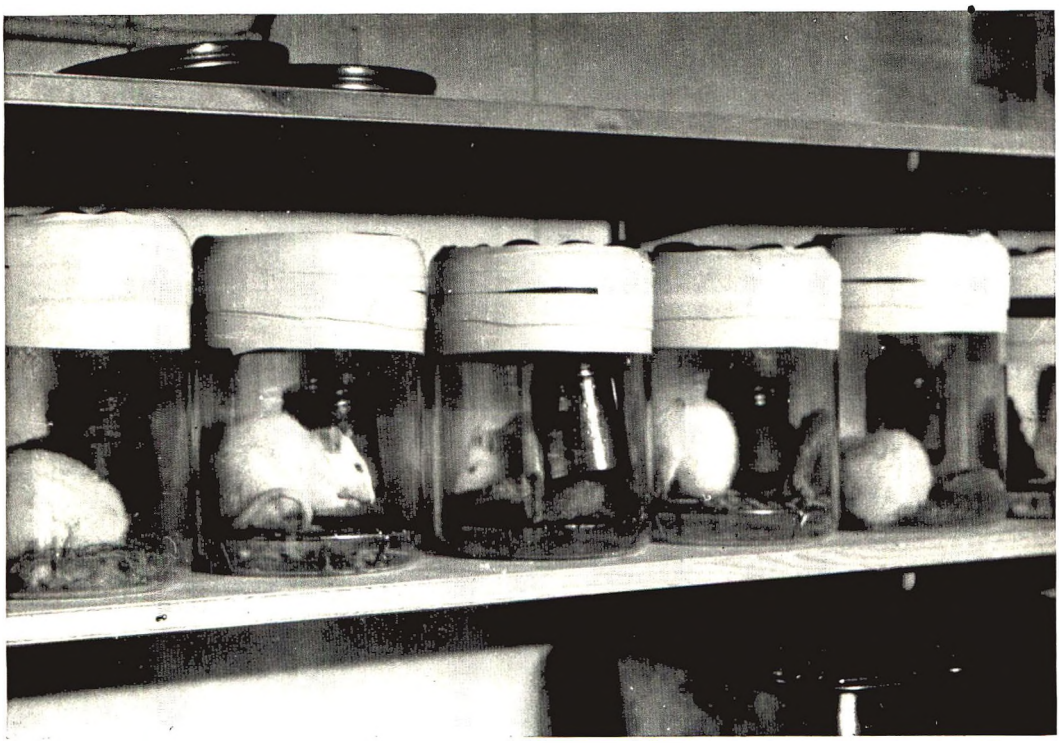

Fig. 4B Individual germfree isolators in the conventional animal room. The rubber sleeve protecting the filter was wrapped with tape as a further protective measure.

TABLE 4

Effect of monocontaminations in single vitamin K-deficient germfree rats

Type of microorganism[1]	Source	Prothrombin activity values after 48 hours[2]
		%
Sarcina	rat oral and enteric strain	100
Escherichia coli	rat enteric strain	100
Lactobacillus acidophilus	rat oral strain	< 10
Diphtheroid	rat oral strain	< 10
Sporeformer	rat enteric strain	< 10
Bacteroides I	rat enteric strain	< 10
Bacteroides II	rat enteric strain	< 10
None	—	< 10

[1] Each animal received the equivalent of 0.1 ml of a 24-hour broth culture which was washed and resuspended in saline.
[2] Initial prothrombin activity < 10%.

nose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, and xylose were not fermented. Esculin, α-methyl glucoside and α-methyl mannoside were not hydrolyzed. Litmus milk was slowly reduced and acidified with the formation of a soft coagulum. The methyl red, Voges-Proskauer and catalase tests were positive. Nitrate was reduced to nitrite and hippurate was hydrolyzed. The organism did not produce indole or ammonia from peptones or possess gelatinase activity. Blood was not hemolyzed. The organism does not correspond closely to any of the mem-

bers of the family *Micrococcaceae* listed in the current edition of Bergey's Manual of Determinative Bacteriology (Breed et al., '57). This, or very similar organisms, have been frequently encountered by us in the oral cavity and intestinal tract of conventional rats. When germfree rats are brought into a conventional environment, this type appears to be among the first organisms to become established in the animals. The active strain of *E. coli* was isolated from rat feces and gave reactions typical of this species in the usual presumptive and confirmatory tests for coliform organisms based on lactose fermentation, nature of growth on eosin-methylene blue agar, and the indole, methyl red, Voges-Proskauer and citrate tests. It has not been further studied.

DISCUSSION

It is clearly apparent from the results of the present study that both the Swedish and Lobund strains of germfree rats require an exogenous source of vitamin K in the germfree state. Both strains were equally susceptible to vitamin K deficiency symptoms when maintained with a properly purified vitamin K-deficient diet.

That the animals were actually suffering from vitamin K deficiency was shown by the curative effect of subsequently administered vitamin K_1. Menadiol tetrasodium diphosphate and menadiol sodium sulfate may be substituted for vitamin K_1 but they are considerably less efficient. Vitamin K_1 at a dose of 25 μg/kg of body weight could reverse the hypoprothrombinemia in germfree rats. This compares with the observation of Dam and Søndergaard ('53) that 30 μg/kg returned the prothrombin time to normal in 20 hours in vitamin K-deficient chicks. In the present study menadiol tetrasodium diphosphate was approximately one tenth as effective, and menadiol sodium sulfate one-hundredth as effective as vitamin K_1 in restoring the prothrombin levels of the vitamin K-deficient germfree rat to normal.

These observations are in accord with the well-recognized fact that these compounds are also less effective in dicoumarol poisoning than vitamin K_1. It has also been reported (Mushett and Seeler, '47) that vitamin K_1 was up to 250 times as effective as menadione in the treatment of sulfonamide-induced hypoprothrombinemia. Frost et al. ('56), however, found menadione sodium bisulfite to be superior to vitamin K_1 in treating chickens with a vitamin K deficiency which was uncomplicated by the use of sulfonamides or dicoumarol. Quick and Collentine ('51) found that in cholecystonephrostomized dogs menadione, in doses up to 70 μg/kg of body weight, could not raise the prothrombin level to more than 40% of normal, whereas vitamin K_1 increased it to normal at doses of 9 μg/kg. On the other hand, Quick et al. ('54) found the menadione sodium bisulfite complex to be more active than vitamin K_1 in the same test system. Vitamin K_1 has been shown to increase the oxidative phosphorylation of liver mitochondria, whereas menadione was inactive or even inhibitory (Martius and Nitz-Litzow, '51). Differences in the biological activity of menadiol tetrasodium diphosphate and menadiol sodium sulfate may be of interest in view of the different chemical oxidation pathways found for these two compounds by Clark et al. ('58).

Although there was apparently no difference in susceptibility to vitamin K deficiency between the Lobund and Swedish strains of germfree rats fed the purified diet used, there is no doubt that the males of both strains were more susceptible than the females. The difference was most pronounced in the case of the peanut oil-containing diet with which all the females survived the full 120-day experimental period and all the males succumbed by the seventy-fifth day. This suggests that the peanut oil used might contain sufficient vitamin K to sustain the females but not the males. It recalls experiments in which the feeding of irradiated beef to rats resulted in the death of all the males with vitamin K deficiency symptoms, whereas the females survived (Metta et al., '59).

In contrast with peanut oil which exhibited some sparing effect on vitamin K-deficient animals, lard appeared to potentiate the effects of vitamin K deficiency. The available information does not permit an explanation of the mode of action

of the lard. However, these observations may assume added importance when regarded in the light of reports that heated or unheated fats of animal origin (Nightingale et al., '47; Kraybill, '59)[5] or irradiated meat incorporated in vitamin K-deficient diets of conventionally reared rats resulted in the appearance of vitamin K deficiency symptoms (Metta et al., '59).

The demonstration that monoinfection of vitamin K-deficient germfree rats with either *E. coli*, or a sarcina-like micrococcus, completely reversed the vitamin K deficiency symptoms is consistent with the earlier work of Almquist ('41) and Jacobsen and Dam ('60), who found that similar organisms, among others, contained compounds with vitamin K activity. However, we believe the present study represents the first unequivocal demonstration that an experimentally induced vitamin deficiency may actually be reversed by colonization of the host animal by a single strain of bacteria.

SUMMARY

1. Vitamin K deficiency was readily induced in two different strains of germfree rats raised with a vitamin K-deficient diet. Conventional animals fed the same diet showed no deficiency symptoms.

2. The male germfree rats were more susceptible to vitamin K deficiency than the females.

3. The addition of 25% of lard to the basal diet accelerated the appearance of vitamin K deficiency symptoms, whereas 10% of peanut oil in the diet exerted a sparing effect.

4. Vitamin K_1 was 10 times more effective than menadiol diphosphate and 100 times more effective than menadiol sodium sulfate in curing the vitamin K deficiency symptoms.

5. Monoinfection of germfree vitamin K-deficient rats with *Escherichia coli* or a sarcina-like micrococcus isolated from conventional rats reversed the vitamin K deficiency within 24 to 48 hours.

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