Further Evidence of an Unidentified, Growth-Promoting Factor (MGF) in Cows' Milk[1]

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The results of research on unidentified growth factors required by the chick, reported after the discovery of vitamin B_{12} and preceding 1956, have been reviewed by Cheldelin and King ('54), Plaut and Betheil ('56) and anonymously ('52, '56). More recently Dam et al. ('59) presented evidence, based on studies with chicks fed a purified diet deficient in zinc, of the existence of an unidentified organic growth factor in liver extract. This appeared either to replace zinc to a great extent, or render it more available, as well as promote an increased rate of gain. Another organic factor, present in a mixture of distiller's dried solubles, dried whey and fish solubles, appeared to be only growth-promoting in experiments with chicks fed a purified diet adequate in zinc. Kratzer et al. ('59), using turkey poults fed a purified diet, reported the presence in soybean oil meal of an organic, methanol-soluble growth-promoting factor. Wilcox et al. ('61) obtained increased growth in turkey poults when the diets contained a water-soluble extract of soybean oil meal. Atkinson and Kratzer ('60) found in studies with chicks fed a purified diet that liver extract contains a growth-promoting factor, in confirmation of previous work. In addition they obtained evidence of a factor affecting size of spleen. As a consequence of these reports many research workers have generally accepted the presence in crude materials of at least two, and perhaps three, unidentified organic growth factors. Because of lack of progress in isolating any of these factors, however, and the excellent growth frequently observed in chicks fed so-called purified diets, some doubt has been cast on the existence of unidentified factors, and the hypothesis of imbalance of known nutrients has been offered as an explanation of the variable results obtained in experiments on unidentified growth factors.

In further studies of unidentified growth factors, however, results have been obtained that render this hypothesis invalid, because they clearly demonstrate the existence in cows' milk of an unidentified factor that promotes chick growth. This factor has been previously designated the whey factor by Hill ('48) and other research workers. In addition the results of these studies provide a reasonable explanation for the failure in many instances to obtain any significant response to the milk growth factor (MGF).

EXPERIMENTAL

The chicks in experiment 1 were Vantress × Barred Plymouth Rock chicks of mixed sex. Arbor Acres Farm day-old, male White Plymouth Rock chicks were used in all other experiments. Each lot contained 15 chicks at the start, with two lots being subjected to each treatment. The chicks of experiment 1 were housed in uncoated Petersime batteries. The chicks of all other experiments were housed in resin-coated Petersime batteries, equipped with resin-coated wire-mesh floors and resin-coated water pans and feeders. Each chick was identified with a numbered wingband, weighed individually at the start and weekly thereafter. The duration of each experiment was 4 weeks. Feed and water were supplied ad libitum and feed consumption was recorded at the time of weighing. A few chicks died during the experiments but the number was not related to treatment.

The composition of the basal diet is given in table 1. All known vitamins,

Received for publication October 13, 1961.
[1] This investigation was aided in part by a grant from Foremost Dairies, Inc., San Francisco, California, to the University of California, and by the use of some of the facilities of Foremost Dairies Central Research Laboratories.

TABLE 1
Composition of basal diet

	%
Glucose[1]	53.1
Isolated soybean protein	28.5
Corn oil[2]	7.5
Cellulose[3]	3.0
DL-Methionine	0.75
Glycine	0.35
Mineral mixture[4]	6.07
Vitamin mixture[5]	0.78
Butylated hydroxytoluene	0.025

[1] Cerelose, Corn Products Company, New York.
[2] Mazola, Corn Products Company, New York.
[3] Solka-Floc, Brown Company, Berlin, New Hampshire.
[4] When included in the diet at a level of 6.07%, the mineral mixture contributed the following elements in amounts per kg of diet: (in grams) calcium, 12.0; phosphorus, 7.0; potassium, 4.0; chlorine, 3.86; sodium, 2.5; and (in milligrams) magnesium, 520.0; manganese, 100.0; zinc, 50.0; iron, 30.0; copper, 4.5; iodine, 2.0; molybdenum, 0.5; cobalt, 0.25.
[5] When included in the diet at a level of 0.78%, the vitamin mixture contributed the following vitamins in amounts per kg of diet: (in milligrams) choline·Cl, 1,500.0; inositol, 250.0; niacin, 50.0; Ca pantothenate, 20.0; pyridoxine·HCl, 6.0; riboflavin, 6.0; thiamine·HCl, 4.0; folic acid, 2.0; menadione, 0.5; biotin, 0.2; α-tocopheryl acetate, 20.0; also vitamin B_{12}, 20.0 μg; vitamin A, 5,000 IU, vitamin D_3, 400 ICU plus 5.3 gm of vitamin A, D and E diluents.

essential amino acids and essential minerals were provided in the diet at levels in excess of the requirements reported by the National Research Council ('60). The protein content of the diet was 25.2%. The isolated soybean protein used in the diet was for the most part ADM C-1 Assay Protein[2] but Promine[3] was used in several experiments. In experiment 15 isolated soybean protein prepared for defatted, unheated soybean flakes in the pilot plant laboratory of Foremost Dairies, Inc.[4] was used. This experimental protein was extracted with ammonium hydroxide at pH9.2, and the ammonia driven off the solution by heating. After this the pH was lowered rapidly to pH2 with hydrochloric acid. The protein solution was then divided into two portions, one of which was treated with activated charcoal. The charcoal was removed by filtration and the pH of both solutions raised to pH4.3, the isoelectric point of soybean protein, with calcium hydroxide. The precipitate was separated by centrifugation and tray-dried at low temperatures after being heated sufficiently to destroy any residual anti-trypsin.

The dried skim milk, dried whey and MGF concentrates used in the investigation were also prepared in the pilot plant laboratory of Foremost Dairies, from fresh products obtained locally. The milk was found to contain 34.4% of protein, 49.6% of lactose hydrate, 7.3% of ash and 5.2% of moisture. The several supplies of dried whey used in the experiments contained on the average, 13.0% of protein, 71.6% of lactose hydrate, 8.1% of ash and 4.5% of moisture. Milk growth factor concentrate C-1, prepared from whey W-1 contained 3.1% of nitrogen, 44.6% of lactose hydrate, 20.9% of ash and 2.3% of moisture. It contained 27.2% of the lactose hydrate present in the original whey.

MGF concentrate C-1 was prepared by heat precipitation of lactalbumin at pH5.2 to 5.8 and removal by centrifugation, concentration of the filtrate with the resultant precipitation of much of the lactose and removal by centrifugation. The final filtrate was then concentrated and dried. Assuming little loss of MGF on the precipitates, the amount of the factor in the concentrate was approximately 2.35 times that in an equivalent amount of dried whey, made from a portion of the original supply of liquid whey.

MGF concentrate C-2, made from whey W-2, was prepared in much the same manner as concentrate C-1 except that before concentration and drying the final filtrate was passed over cation and anion exchange resins. This removed some nonprotein nitrogen as well as most of the cations and anions. The final dried product contained 2.8% of nitrogen, 75.2% of lactose hydrate, 2.0% of ash and 3.4% of moisture. The concentration of MGF in this preparation was caculated to be 2.75 times that in the original, when dried. It contained 26.9% of the lactose hydrate present in the original whey.

MGF concentrate C-4, made from whey W-4, was prepared by removal of lactalbumin and the greater portion of the minerals and represent a calculated concentration of MGF of 1.2 times that in the original whey, when dried. It contained 0.72% of nitrogen, 90.1% of lactose hydrate, 5.0% of ash and 1.1% of moisture.

[2] Archer-Daniels-Midland Company, Cincinnati.
[3] Central Soya Company, Inc., Chicago.
[4] Foremost Dairies Central Research Laboratories, Petaluma, California.

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

RESULTS AND DISCUSSION

This investigation was initiated by a study of fractions of milk, prepared in the pilot plant laboratory of Foremost Dairies from the same supply of liquid whey. The fractions were lactalbumin, lactose hydrate, and the concentrated and dried filtrate (MGF concentrate) remaining after removal of these precipitates. The results of this experiment are presented in table 2. In this experiment significant responses over the basal group ($P < 0.01$) were obtained when those groups of chicks were fed dried whey or MGF concentrate or combinations of MGF concentrate with either lactose hydrate or lactalbumin or both these substances. The responses to lactose hydrate, lactalbumin and lactose hydrate plus lactalbumin were not significant. The MGF concentrate fed in the experiment provided 0.95% of lactose hydrate. The results of the experiment indicated, therefore, that the MGF concentrate contained an unidentified nutrient, not present in appreciable quantities in the lactalbumin or lactose hydrate fractions, which promoted chick growth.

Studies of unidentified growth factors using the chick as the experimental subject have been complicated by the extremely variable responses obtained in repeated experiments. This proved to be the major difficulty in continuing the investigation of MGF. The response of chicks to this factor varied in 29 studies of dried whey and other milk fractions from 2.2 to 25.3% with a mean response of 11.6%. The responses were all positive and statistically highly significant ($P < 0.0005$). The differences in gains between experiments were equally significant. No interaction between diets and experiments was found to exist.

The average maximal weight attained by all of the Arbor Acre chicks fed the basal diet supplemented with 5% of dried whey was 568 gm and that attained by the chicks fed MGF concentrate, equivalent to 5% of dried whey, was 573 gm. The average maximal weight of all groups was 571 gm. When the average weight of chicks fed the unsupplemented basal diet approached that of chicks supplied with MGF, the chicks obviously received very nearly adequate supplies of this factor from sources other than dried whey and MGF concentrates. This is illustrated by figure 1 in which is plotted the regression of the percentage gain in weight of the chicks, supplied MGF preparations, on the weight in grams of chicks fed the unsupplemented basal diet. The values in experiment 1, using Vantress × Barred Plymouth Rock chicks, have been adjusted so that they are equivalent to weights attained by the groups in which Arbor Acre chicks were used. The regression is linear and the percentage gain in weight is inversely correlated with the weight of the chicks fed the unsupplemented basal diet. The in-

TABLE 2

Results showing relative milk growth factor (MGF) content of equivalent amounts of whey and whey fractions (exp. 1)

Treatment	Av. weight at 4 weeks	Gain over basal	Feed/ gain
	gm	%	
Basal diet	362	—	1.84
+ dried whey W-1, 5%	441	24.6	1.57
+ lactalbumin, 0.36%	380	5.6	1.84
+ lactose hydrate, 2.73%	389	8.4	1.73
+ MGF concentrate C-1, 2.12%[1]	444	25.5	1.51
+ lactalbumin, 0.36%, and lactose hydrate, 2.73%	388	8.1	1.87
+ lactalbumin, 0.36%, and MGF concentrate, 2.12%	434	22.4	1.56
+ lactose hydrate, 2.73%, and MGF concentrate, 2.12%	446	26.2	1.66
+ lactose hydrate, 2.73%, lactalbumin, 0.36%, and MGF concentrate, 2.12%	461	30.8	1.56

[1] MGF concentrate C-1, prepared from whey W-1, supplied 0.95% lactose hydrate.

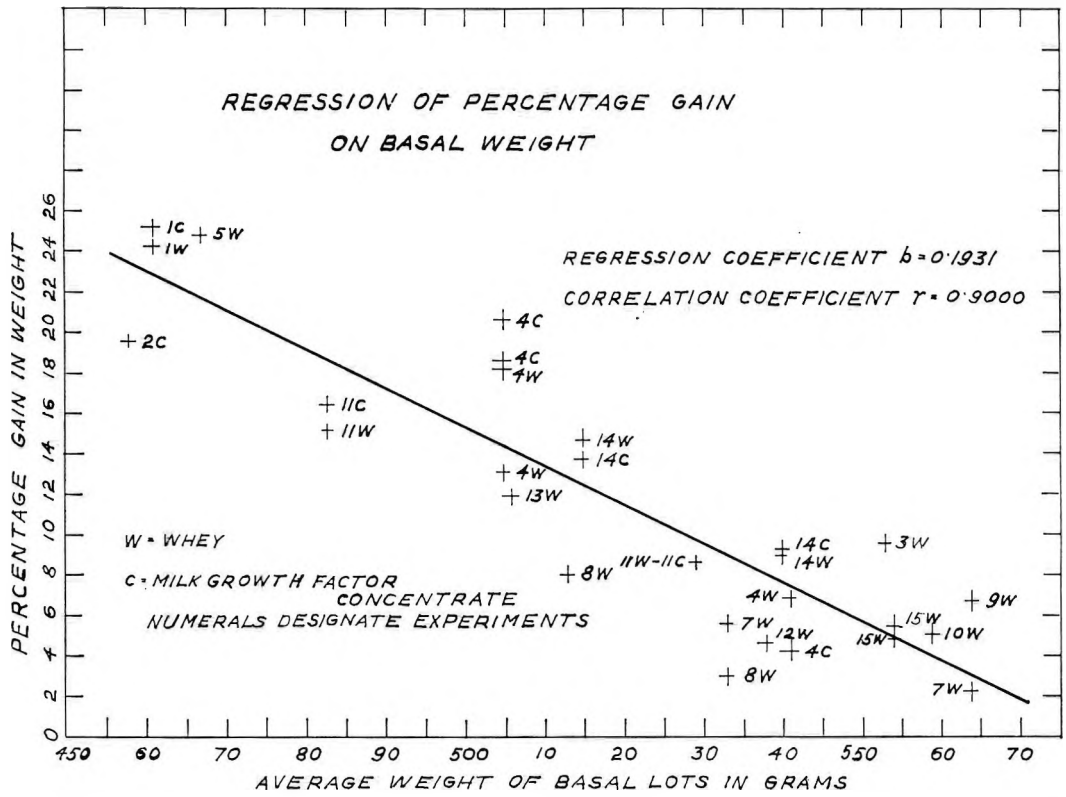

Fig. 1 Regression of percentage gain of chicks on the average weight of the lots fed the basal diet.

verse correlation between these values was excellent ($r = 0.90$).

Some evidence of seasonal effect on response to MGF was observed in this work. In 5 experiments conducted during the period April to August, 1960, the response range varied from 2.2 to 8.0%; and in the period, September to March, it varied from 4.2 to 25.2%. However, with one exception the poor responses during this period were obtained with Promine in two experiments conducted in February, 1960, and January, 1961. The average of the responses was 7.35%. In contrast, the average response in these experiments with ADM assay protein was 15.0%. The difference in the response obtained with these types of isolated soybean protein was significant ($P < 0.05$).

The variability of isolated soybean protein in amount of the milk growth factor was revealed in a third experiment in which ADM assay protein and Promine were compared with laboratory-prepared, isolated protein. The results of this experiment are presented in table 3. The responses obtained with the two laboratory-prepared proteins were approximately the same and averaged 12.6%, whereas the responses obtained with ADM assay protein and Promine were 4.9 and 5.4%, respectively. The difference in the responses obtained with the laboratory-prepared proteins and the commercial ones was highly significant ($P < 0.01$). The procedure for making the laboratory-prepared isolated soybean protein used in this experiment is the only reasonably successful one developed in attempts to produce isolated soybean protein that is more nearly free of MGF. The results of these attempts, however, indicated that MGF is heat-stable, resistant to oxidation and not efficiently adsorbed by activated charcoal in solutions of soybean protein at pH2, pH6 and pH9.0 to 9.2.

TABLE 3

Results showing difference in gain obtained with dried whey using commercial and laboratory-prepared isolated soybean protein in the basal diet (exp. 15)

Treatment	Av. weight at 4 weeks	Gain over basal	Feed/ gain
	gm	%	
Basal diet, ADM C-1 Assay Protein[1]	554	—	1.54
+ dried whey W-5, 5%	579	4.9	1.54
Basal diet, Promine[2] protein	554	—	1.53
+ dried whey W-5, 5%	581	5.4	1.57
Basal diet, laboratory-isolated protein	513	—	1.54
+ dried whey W-5, 5%	576	13.3	1.49
Basal diet, laboratory-isolated protein, charcoal-treated	517	—	1.53
+ dried whey W-5, 5%	573	11.8	1.50

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

The type of carbohydrate used in the basal diet was found to affect the rate of gain in one of two experiments in which sucrose was compared with glucose monohydrate. In the first experiment the response to dried whey with sucrose as the carbohyrate was 5.6% and with anhydrous glucose, 2.2%. This difference in response was obviously of no significance. The results of the second experiment are given in table 4. In this experiment the response to dried whey with anhydrous glucose was 15.6% and that with sucrose 8.7%. This difference proved to be highly significant ($P < 0.01$). Better responses were also obtained with sucrose and MGF than with glucose and this factor, indicating either that an insufficient amount of the factor was supplied in the experiment or that the supply of sucrose used in this experiment contained an unidentified growth-promoting factor not identical with MGF.

The variable response to MGF, demonstrated in figure 1, may therefore be due to both a seasonal effect and variation in the amount of the factor in the ingredients of diet, particularly the isolated soybean protein.

The more striking results obtained with 5% of dried whey and MGF concentrates equivalent to 5% of dried whey are presented in table 5. These results were obtained in the period from September to March. The average gain in weight promoted by dried whey was 18.4% and that obtained with MGF concentrate was 19.0%. The increased gain promoted by these milk fractions in all experiments was highly significant ($P < 0.0005$).

Because of the failure to obtain a significant response with lactose hydrate in experiment 1, studies were undertaken to determine whether reagent grade lactose hydrate was free of the factor. Therefore

TABLE 4

Results showing reduction in gain obtained with dried whey and an equivalent amount of milk growth factor concentrate by replacing glucose with sucrose in the basal diet

Treatment	Av. weight at 4 weeks	Gain over basal	Feed/ gain
	gm	%	
Promine basal diet, glucose	483	—	1.65
+ dried whey W-4, 5%	551	15.1	1.61
+ MGF concentrate C-4, 4.2%[1]	554	16.0	1.54
Promine basal diet, sucrose	529	—	1.52
+ dried whey W-4, 5%	572	8.8	1.51
+ MGF concentrate C-4, 4.2%	572	8.8	1.51

[1] MGF concentrate C-4 was prepared from whey W-4.

TABLE 5

Results showing comparative milk growth factor (MGF) content of equivalent amounts of whey and MGF concentrate

Exp. no.	Av. weight at 4 weeks			Gain over basal	
	Basal diet	Plus 5% dried whey	Plus equivalent MGF concn.	Whey	MGF concn.
	gm	*gm*	*gm*	%	%
1	362	441(W-1)	444(C-1)[1]	24.6	25.5
2	458	—	541(C-1)	—	19.6
4a	505[2]	590(W-1)	600(C-1)[1]	18.2	20.5
4b	505[2]	566(W-2)	591(C-2)[1]	13.1	18.6
5	467	573(W-3)	—	24.9	—
11	483	551(W-4)	554(C-4)[1]	15.1	16.0
14	515	579(W-5)	579(C-4)	14.6	13.7
Av.	471	550	551	18.4	19.0

[1] MGF concentrate C-1 was prepared from W-1, C-2 from W-2 and C-4 from W-4.
[2] Basal groups were common to experiments 4a and 4b.

TABLE 6

Results of experiments on milk growth factor (MGF) effect of lactose hydrate[1]

Exp. no.	Av. weight at 4 weeks			Gain over basal	
	Basal diet	Plus 5% dried whey	Plus equivalent lactose	Whey	Lactose
	gm	*gm*	*gm*	%	%
3	553	601	592	9.4	7.5
5	467	573	574	24.9	25.1
7	564	575	564	2.2	−0.8
13	506	561	545	11.9	8.4
Av.	523	578	569	12.1	10.1

[1] Baker Analyzed Reagent Lactose Hydrate, Baker Chemical Company, Phillipsburg, New Jersey.

analyzed reagent grade[5] lactose was studied. The reported analysis of this lactose (in per cent) was: insoluble matter, 0.003; residue after ignition, 0.03; dextrin, 0.0005; starch, 0.0005; sucrose, 0.01; glucose, 0.05; heavy metals as lead, 0.0003; and iron, 0.0003. The results of 4 experiments are given in table 6. The quantity of lactose added to the basal diet was 3.5%. This was approximately equivalent to the amount of lactose in 5% of dried whey. Contrary to expectations, reagent grade lactose hydrate promoted an average gain of 10.1% whereas that obtained with dried whey was 12.1%. The improved growth obtained with lactose hydrate and dried whey was highly significant ($P < 0.005$) but the difference in gains obtained with these products was not significant.

The response obtained with reagent grade lactose hydrate does not appear, however, to be due to lactose per se. In experiment 1, 2.73% of lactose hydrate failed to promote a significant gain in weight over the basal group whereas the MGF concentrate C-1 which provided 0.95% lactose hydrate resulted in a highly significant response.

In another experiment in which MGF concentrate C-2 was fed, 0.91% of the dried material promoted a final average weight of 582 gm. This was slightly greater than the final average maximal weight of all groups in the investigation receiving adequate amounts of dried whey or MGF concentrate, and represented an increased gain of 16.6% over the basal group. The response was highly significant ($P < 0.005$). The quantity of lactose hydrate supplied by the MGF concentrate was 0.68%.

In two experiments in which graded levels of dried whey were fed, the average responses to 2.0, 3.5, and 5.0% of dried whey were 10.6, 12.9, and 17.2, respec-

[5] Baker Analyzed Reagent Grade Lactose, Baker Chemical Company, Phillipsburg, New Jersey.

tively. The average maximal response in both experiments was 582 gm. This was obtained only when 5% of dried whey which supplied approximately 3.5% of lactose hydrate was included in the basal diet. These observations on the effectiveness of different levels of lactose supplied in the concentrates provide further evidence that lactose hydrate per se is not MGF. But it seems highly probable that under certain conditions of lactose precipitation, MGF is either adsorbed on the surface of lactose hydrate crystals or occluded in them during crystal formation, or both. An experiment was conducted to determine the chick response to MGF in dried skim milk by feeding three levels of dried skim milk and equivalent levels of dried whey. The results of this experiment are presented in table 7. The greatest response was obtained with 11.01% of dried skim milk ($P < 0.025$), while the greatest response to dried whey was obtained at an equivalent level (8%) although this was statistically less significant ($P < 0.10$).

MGF is water-soluble, reasonably heat-stable, not efficiently adsorbed by activated charcoal and not adsorbed by cation and anion exchange resins during demineralization of MGF solutions by a procedure in which considerable quantities of nonprotein nitrogen are removed. It does not appear to be identical with any known nutrient. Identity with known B-group vitamins is ruled out by the results of 4 experiments in which the quantity of these vitamins in the diet was doubled. No improvement in gain was obtained by increasing B-group vitamins, but the increased gain of 7.4% obtained with 5% of dried whey was highly significant ($P < 0.01$).

Identity of MGF with any of the minerals present in whey also appears to be ruled out. MGF concentrate C-2, which promoted a highly significant increased gain when fed at a level of 0.91%, contained 2.0% minerals. The quantity of minerals furnished by the concentrate was thus only 0.0182%. The reagent grade lactose studied in the investigation contained 0.03% of nonignitable material. At the level of 3.5% of lactose included in the basal diet, this quantity provided 0.00105% of mineral matter. Moreover, in an experiment in which whey ash, prepared in a manner that minimized loss of minerals subject to sublimation, was fed at a level equivalent to 5% of dried whey, no increase in gain was obtained with the ash. In contrast, 5% of dried whey promoted an increased gain over the basal of 9.5% ($P < 0.20$).

Finally, identity of MGF with any essential amino acid appears highly improbable, since in the experiment in which 0.91% of MGF concentrate C-2 promoted a significant response over the basal group of 16.6%, the amount of protein supplied (mostly nonprotein nitrogen) was only 0.18%. The possibility that this quantity of added dietary protein would be biologically measurable is extremely remote. Hill ('48) concluded from a series of experiments that the factor contained in dried whey is also present in variable amounts in soybean oil meal. He demonstrated that dried whey was effective in promoting growth in chicks, but the response varied with the sample of soybean oil meal used in the diet. The present work supports the findings of Hill, and indicates that isolated

TABLE 7

Results showing difference in gain obtained with dried skim milk and equivalent amounts of dried whey

Treatment	Av. weight at 4 weeks	Gain over basal	Feed/gain
	gm	%	
Promine basal	568	—	1.51
+ dried skim milk, 2.75%	572	0.9	1.59
+ dried skim milk, 6.88%	576	1.5	1.55
+ dried skim milk, 11.01%	616	9.2	1.53
+ dried whey W-5, 2%	581	2.6	1.53
+ dried whey W-5, 5%	593	4.7	1.56
+ dried whey W-5, 8%	599	6.0	1.58

soybean protein also influences the chick's response to dried whey.

SUMMARY

Repeated studies have been made of an unidentified growth factor in milk products (MGF), using the chick as the experimental subject. The factor increased rate of gain significantly ($P < 0.0005$), but the response during the investigation varied from 2.2 to 25.3% with a mean response of 11.6%. In the initial experiment, lactalbumin, lactose hydrate and a combination of these substances promoted a slight response, whereas a marked response was obtained from dried whey and MGF concentrate prepared from the same batch of liquid whey. The variable response appeared to be caused in part by seasonal conditions and in part to the presence of variable quantities of the factor in the soybean protein used in the basal diet. The results of two experiments indicated that at times the carbohydrate used in the basal diet influenced the response of the chicks. Dried skim milk was as effective in improving growth as an equivalent level of dried whey.

No improvement in gain was obtained by doubling the quantity of B-group vitamins in the basal diet or by supplementing the diet with ash of dried whey prepared in a manner to minimize loss of minerals by sublimation. The character of the basal diet and the limited amount of protein (mostly nonprotein nitrogen expressed as protein) added by the MGF concentrates ruled out the possibility of amino acid deficiency. Contrary to expectations, analyzed reagent grade lactose hydrate was found to promote increased chick gain significantly but the evidence indicated this was not due to lactose per se. MGF is water-soluble, heat-stable, resistant to oxidation, not efficiently adsorbed by activated charcoal, and not adsorbed by cation and anion exchange resins during the removal of basic and acidic groups from liquid whey.

ACKNOWLEDGMENTS

The counsel and technical assistance of D. D. Peebles, Associate, University of California and of Paul Clary, M. R. Lambert and C. A. Kempf of Foremost Dairies, Inc. are gratefully acknowledged.

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Protein Synthesis in Vitamin B_6 Deficiency in the Rat[1]

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In vitamin B_6 deficiency in the rat there is an excess production of urea (Hawkins et al., '46; Caldwell and McHenry, '53; Hawkins et al., '59). This vitamin is the precursor of a coenzyme that functions in several systems involving the nonoxidative metabolism of amino acids. When these systems are impaired there may therefore be an increased oxidation of amino acids. This could account for the increased production of urea. It is also possible that anabolic processes in protein metabolism are impaired. The experiment described in this paper was an investigation of that possibility.

Since the liver is particularly active in the production of protein, it seemed an appropriate organ to study for this purpose. The protein content of the liver was measured in rats showing impairment of growth from deprivation of vitamin B_6. This was done before and after a short period of rapid protein repletion following the withdrawal of protein from the diet. The same studies were made on the protein of blood plasma.

EXPERIMENTAL PROCEDURE

The percentage composition of the diets was as follows: vitamin-test casein, 0, 16, and 40, with corresponding amounts of sucrose at 85, 69, and 45; cellulose flour, 4; corn oil, 7; salt mixture (Wesson, '32), 4. Vitamins were added to the diets and given to the rats by injection in the manner and amounts described in a previous paper (Hawkins et al., '59).

Young rats of both sexes, and with an average individual body weight of 87 gm were arranged into three groups, also as previously described (Hawkins et al., '59): those in groups A and B were given vitamin B_6, and those in group C were not. The last group showed varying degrees of anorexia during the course of the experiment, and impaired growth when compared with the animals of group A, which were maintained with the same amount of food. The animals of group B were given smaller amounts of food, to keep their weights similar to those of group C. All were maintained individually in screen-bottom cages. Water was supplied ad libitum.

The rats were fed the diet containing 16% of protein for 65 days, at which time the mean body weight of those in group A had increased by 112%, and of those in the other two groups by 64%. At this stage about one-third of the animals in each group were killed. The rest were deprived of dietary protein for 25 days, then half of them were killed. Those that remained were killed after a repletion period of three days on the diet containing 40% of protein. Animals were killed with pentobarbital and ether.

Samples of blood were taken from the hearts, oxalated, and the plasma separated. Livers were removed and weighed. The liver was homogenized with 10% trichloroacetic acid solution, in proportions of about one to 10, and the nitrogen content of the whole homogenate and of its filtrate determined by a micro-Kjeldahl procedure. The difference between the two values was taken as protein nitrogen. The protein in blood plasma was determined by a biuret method (Gornall et al., '49), and the nonprotein nitrogen by nesslerization of digested tungstic acid filtrate.

RESULTS

The results are shown in table 1.

The level of protein in the blood plasma was not affected by the vitamin B_6 deficiency. The nonprotein nitrogen showed a tendency toward higher values, which

Received for publication October 23, 1961.
[1] Issued as National Research Council no. 6754.

TABLE 1

Nitrogen in the liver and the blood plasma of rats with, or deprived of, vitamin B_6, and the effect of protein depletion and repletion[1]

	Group[2]	After fed a diet with 16% protein for 65 days	After 25 days without protein	After fed a diet with 40% protein for 3 days
Liver, % of body weight	A	2.8—3.7 3.2 ± 0.25 (22)	3.2—4.8 3.9 ± 0.48 (22)	3.3—4.8 4.0 ± 0.38 (22)
	B	2.5—3.8 2.9 ± 0.24 (21)	3.2—5.8 3.9 ± 0.67 (20)	3.0—4.8 3.9 ± 0.42 (22)
	C	2.3—6.8 3.4 ± 0.70 (22)	2.6—5.2 3.6 ± 0.54 (21)	3.2—4.8 3.9 ± 0.40 (20)
Protein N, gm/100 gm	A	2.96—3.91 3.35 ± 0.23 (22)	1.54—2.43 1.99 ± 0.23 (22)	2.71—3.90 3.24 ± 0.22 (23)
	B	2.35—4.49 3.39 ± 0.37 (21)	1.23—2.91 2.17 ± 0.51 (21)	3.03—4.66 3.47 ± 0.31 (22)
	C	1.93—5.10 3.12 ± 0.58 (21)	1.92—3.02 2.33 ± 0.30 (22)	2.17—4.03 3.30 ± 0.37 (20)
Nonprotein N, mg/100 gm	A	155—199 184 ± 11.5 (22)	122—212 154 ± 16.6 (22)	163—282 210 ± 22.2 (23)
	B	177—368 227 ± 18.5 (21)	134—197 163 ± 14.6 (21)	191—347 219 ± 28.8 (21)
	C	139—192 177 ± 12.8 (22)	134—218 158 ± 14.3 (22)	138—242 200 ± 20.9 (20)
Plasma protein, gm/100 ml	A	5.5—6.6 6.1 ± 0.20 (21)	3.2—5.2 4.4 ± 0.47 (19)	5.2—6.9 6.0 ± 0.33 (22)
	B	5.4—6.9 6.1 ± 0.29 (20)	3.4—5.6 4.4 ± 0.64 (18)	5.5—6.9 5.9 ± 0.30 (20)
	C	5.2—6.9 6.1 ± 0.33 (19)	3.4—5.3 4.4 ± 0.36 (21)	5.0—6.5 5.7 ± 0.29 (20)
Nonprotein N, mg/100 ml	A	19—31 24 ± 3.6 (21)	14—62 23 ± 8.8 (19)	20—60 40 ± 9.7 (21)
	B	20—39 27 ± 3.8 (20)	21—34 25 ± 3.0 (18)	24—71 41 ± 10.0 (20)
	C	15—45 28 ± 8.1 (19)	15—94 30 ± 13.6 (20)	29—64 45 ± 6.3 (20)

[1] Ranges, averages and standard deviations, with the number of animals indicated in parentheses.

[2] A indicates animals fed the same amount of food as those deprived of vitamin B_6; B, animals fed less food to maintain their body weights the same as those deprived of vitamin B_6; C, animals deprived of vitamin B_6. The only significant difference among A, B, and C was the higher value for nonprotein nitrogen in the liver of the rats in group B at the end of the period when fed the diet with 16% of protein. In that case the P value between B and A or C was < 0.01.

usually occur in this deficiency, and this tendency persisted throughout the periods of protein depletion and repletion, although the differences were never statistically significant.

The liver tended to be larger and its protein concentration lower in the vitamin deficiency, but they were not significantly so, and the differences were not maintained throughout depletion and repletion. The high nonprotein nitrogen in the livers of the inanition control animals (group B) probably was a reflection of the utilization of more amino acids for energy production.

In the vitamin B_6-deficient rats there was no impairment of the capacity for rapid regeneration of the proteins of liver and blood plasma.

DISCUSSION

Beaton et al. ('53), Ross and Pike ('56), and Guggenheim and Diamant ('59) observed significantly enlarged livers in rats fed deoxypyridoxine. Ross and Pike ('56) obtained their information on animals that had just produced young. They also observed at term, in vitamin B_6-deficient rats without deoxypyridoxine, an enlargement of the liver comparable in extent to that shown in our experiment. In all of these experiments when deoxypyridoxine was fed, however, the difference in liver weight relative to body weight was considerable. Guggenheim and Diamant ('59) used two sets of control animals, as we did, and it is important to have the inanition (group B) controls. The experiment of these workers differed from ours in one important respect that was related to the use of deoxypyridoxine: the animals that received it lost weight much more rapidly than ours did from simple deprivation of the vitamin. The food intake of their inanition controls must have been severely restricted, and among them the livers were the largest, which is an important difference from our results. As there are complicating factors, hepatomegaly in vitamin B_6 deficiency in the rat has not been clearly established, as it has in the case of riboflavin deficiency (Guggenheim and Diamant, '59; Mookerjea and Hawkins, '60).

Among the functions of the vitamin B_6 coenzyme in amino acid metabolism is its participation in transamination (Schlenk and Snell, '45; Schlenk and Fisher, '45). A possible role of transamination in protein synthesis has often been considered and discussed (see Linderstrøm-Lang, '39), but experimental evidence for it is not strong. Transaminase activity is high in germinating seeds (Albaum and Cohen, '43; Smith and Williams, '51), but generally it is low in rapidly growing or regenerating mammalian tissue (Cohen and Hekhuis, '41; Bartlett and Glynn, '50a, b). It is reasonable to suppose that in our experiment transamination was impaired in the livers of the vitamin B_6-deficient rats; but there was no impairment of the capacity to regenerate protein in that organ. Beaton et al. ('53) observed that regenerative capacity was not impaired in partially hepatectomized rats given deoxypyridoxine. All of this adds to the evidence that transamination is not important for protein synthesis in the rat.

The most noteworthy result of our experiment was the demonstration that in vitamin B_6 deficiency in the rat the capacity to synthesize some of the metabolically most important proteins of the body was not impaired. This supports the concept that under those conditions an increased catabolism of amino acids reflects a defect in their nonoxidative metabolism and not in anabolic processes.

SUMMARY

Young rats were divided into three groups of the same average body weight. Those of one group were deprived of vitamin B_6. The other two groups served as controls. The animals of one control group were fed the same amount of food as those deprived of the vitamin. Those of the other were maintained at the same rate of growth as the vitamin-deprived animals by restriction of their food intake.

After 65 days, when growth had been significantly impaired by the vitamin deficiency, the protein content of the diet of all animals was adjusted to subject them to successive periods of protein depletion and repletion. At each stage of this regimen the protein and nonprotein nitrogen of the liver and blood plasma were deter-

mined on animals from each of the three groups.

Vitamin B_6 deficiency was not associated at any stage with significant differences among the three groups in the size of the liver, or the concentration of protein in it or in the blood plasma. There was apparently no impairment of the anabolic processes involved in the synthesis of these proteins.

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Observations on Protein Digestion *In Vivo*

IV. FURTHER OBSERVATIONS ON THE GASTROINTESTINAL CONTENTS OF RATS FED DIFFERENT DIETARY PROTEINS[1]

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Rogers et al. ('60) observed that the amount of nitrogen in the contents of the small intestine of rats one to three hours after they had eaten a single meal was greater if the meal contained gelatin or zein, than if it contained casein as the dietary protein. Since the greater accumulation of nitrogen after feeding gelatin or zein was thought to result from a slow rate of cleavage of some of the peptide bonds of these proteins, intestinal contents from rats fed gelatin, zein or an amino acid mixture were fractionated and the distribution of nitrogen among the various fractions was determined. As described below, the distribution was observed to be different for each of the proteins tested.

EXPERIMENTAL

Male albino rats weighing approximately 200 gm were trained to consume their daily ration in a single two-hour interval in a manner similar to that described by Peraino et al. ('59).

Five test diets were used. Four of them contained protein or amino acids,[4] 22%; corn oil, 5%; salts, 5%; and "dextrin" (moist starch heated in an autoclave at 121° for two to three hours, then dried and ground), 68%. The proteins used were casein,[5] zein,[6] and gelatin.[7] The fifth diet ("nitrogen-free") contained corn oil, 5%; salts, 5%; and "dextrin," 90%, but no protein or amino acids. Vitamins were not included in the diets for these short (three-hour) experiments.

For each experiment, a large number of trained rats were fed a single 5-gm test meal in a 30-minute interval, then groups of 5 or 6 rats were killed by chloroform anesthesia at zero time (immediately at the end of the feeding period) and one and three hours later. The stomachs and the small intestines were ligated and then removed. The wet weight and moisture content of the stomach contents were determined as described previously (Harper and Spivey, '58), as also were the dry weight and nitrogen content (Peraino et al., '59).

The intestinal contents were rinsed out with water and diluted to 50 ml. The insoluble residue was separated by slow-speed ($650 \times g$ for 15 minutes) centrifugation. The soluble proteins were precipitated from an aliquot of the supernatant solution by adding 20% trichloroacetic acid (TCA) solution to make the final TCA concentration 5%, and heating the resulting mixture in a boiling water bath until a precipitate formed. The amount of nitrogen in each of the insoluble residues and supernatant fractions was determined by a semimicro Kjeldahl method using mercuric oxide as the catalyst.

In the final experiment groups of rats were fed each of the 5 diets and 3 to 5 rats from each group were killed one hour

Received for publication October 19, 1961.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the National Live Stock and Meat Board, Chicago, Illinois, and Campbell Soup Company, Camden, New Jersey. Some of the crystalline vitamins were kindly provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

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[4] The amino acid diet contained the following amino acids in per cent: L-arginine·HCl, 0.73; L-histidine·HCl, 0.74; L-lysine·HCl, 1.88; L-leucine, 1.20; L-cystine, 0.40; L-tyrosine, 0.60; DL-methionine, 0.80; DL-tryptophan, 0.50; DL-phenylalanine, 1.20; DL-threonine, 1.50; DL-isoleucine, 1.50; DL-valine, 2.10; DL-alanine, 2.00; glycine, 1.00; DL-aspartic acid, 1.00; L-glutamic acid, 5.00; sodium acetate, 0.79.

[5] Borden Company, New York.

[6] Nutritional Biochemicals Corporation, Cleveland.

[7] Kindly provided by Wilson and Company, Chicago.

after the end of the feeding period. The intestines were removed and the contents washed out as described above. The individual fractions were then pooled and the Kjeldahl nitrogen content of each of the pooled fractions was determined. In addition to total nitrogen, amino acid nitrogen (Van Slyke et al., '41) and α-amino nitrogen (indirect formol titration taken as the titer from pH6.0 to pH8.5 by use of a pH meter) were determined on the pooled, TCA-soluble fraction.

The fractionation study was based on the following assumptions: (1) that little nitrogen accumulates in the intestine of the rat unless it is fed a diet containing a protein that is digested slowly or incompletely (Peraino et al., '59; Rogers et al., '60); (2) that after feeding a "nitrogen-free" or a protein-free diet containing crystalline amino acids, any protein in the intestinal contents is of endogenous origin; (3) that insoluble dietary protein and mucosal cells are sedimented by slow-speed centrifugation of the intestinal washings; (4) that protein nitrogen of endogenous secretions remains in the supernatant solution but is precipitable with hot 5% trichloroacetic acid (TCA) solution; and (5) that unabsorbed amino acids and peptides are not precipitated by TCA.

RESULTS

Well-trained rats would eat 5 gm of a test diet containing no protein or 22% of casein or zein in 30 minutes but they would not eat 5 gm of diets containing amino acids or gelatin in this time (see Rogers et al., '60). Therefore, values for the stomach and intestinal contents of rats fed the amino acid or gelatin diets were based on individual intakes of 2.5 to 5.0 gm.

Stomach contents. The rates of stomach-emptying of rats fed the 5 test diets are shown in figure 1. The diet contain-

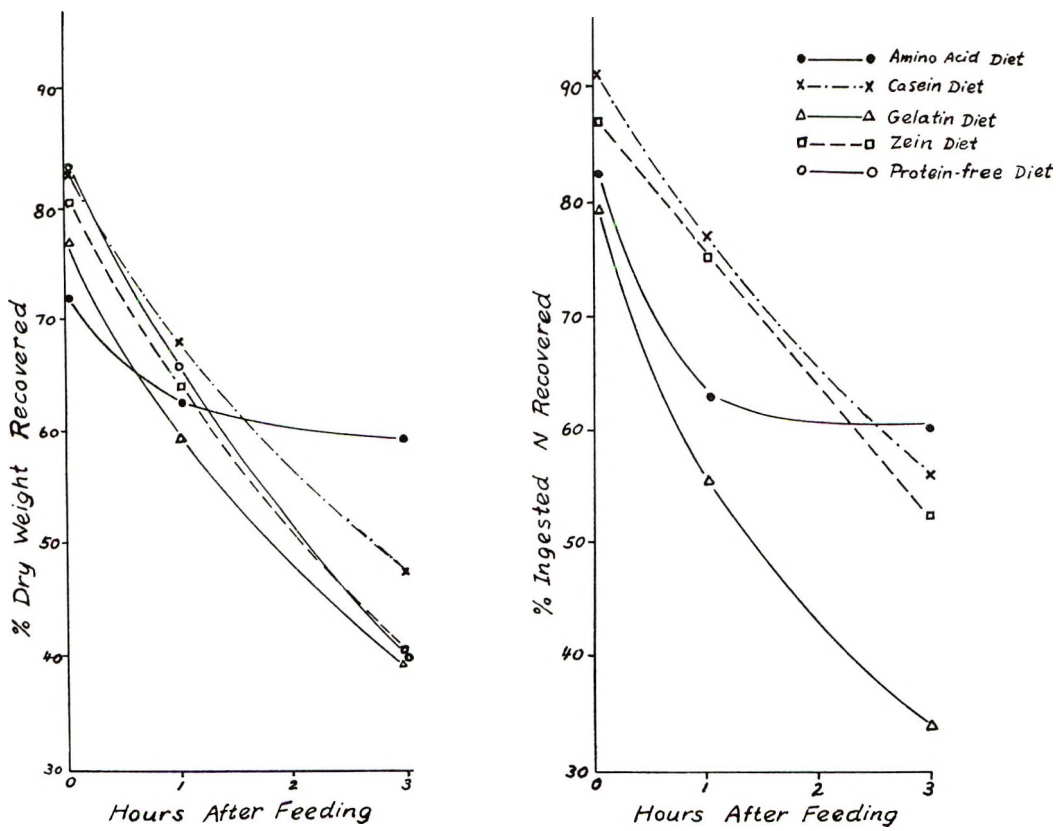

Fig. 1 Rate of emptying of different diets from the stomachs of rats fed a single meal.

ing 22% of zein emptied more rapidly from the stomach than that containing 22% of casein. Although these diets contained 22% of protein as compared with the 30% used in the experiments of Rogers et al. ('60), the effect of the lower protein level on the rate of stomach-emptying in this experiment was small. The curves for percentage of dry weight and for percentage of ingested nitrogen recovered after feeding zein or casein were similar to those obtained previously.

Stomach-emptying curves for rats fed the gelatin and amino acid diets differed from those obtained previously with rats adapted to these two diets. The gelatin diet emptied more rapidly from the stomachs of the nonadapted rats and the amino acid diet emptied more slowly. Nearly 40% of the dry weight of the amino acid diet had passed from the stomach by the end of the first hour after the feeding period; however, the rates of emptying of both dry matter and nitrogen were very slow during the subsequent two hours.

The percentages of moisture in the stomach contents at intervals after feeding the different diets are shown in table 1. The values for the percentage of moisture in the stomach contents of rats that received casein, zein or the "nitrogen-free" diets were considerably lower than those for rats that received the amino acid or gelatin diets. When rats were fed the zein diet the moisture content increased progressively from zero time until the third hour. The values for the percentage of moisture in stomach contents of rats fed the amino acid or the gelatin diets were significantly higher than those for rats fed the other three diets ($P < 0.05$ for zero and one-hour; $P > 0.05$ for three-hours) with values for rats fed the amino acid diet being highest. The stomach contents of rats fed the gelatin and amino acid diets were fluid, whereas those of rats fed the casein, zein and "nitrogen-free" diets formed a compact mass which could be removed intact from the excised stomachs.

Intestinal contents. The amounts of nitrogen in the insoluble residues from the intestinal contents are shown in table 2. Most of the values were relatively low and did not vary greatly at the different time intervals. However, the amount of residual nitrogen in the intestinal contents of rats fed the zein diet increased progressively with time after feeding ($P < 0.001$ as compared with the highest of the other diets). The greatest accumulation occurred three hours after feeding, but the values for the earlier intervals were also several times greater than those for the other groups.

TABLE 1

Moisture content of stomach contents

Diet	0-hours	1-hour	3-hours
	%	%	%
"Nitrogen-free"	62.9 ± 1.9[1]	62.5 ± 1.9[1]	67.9 ± 2.5[1]
Casein	62.5 ± 1.5	63.3 ± 1.3	64.9 ± 1.1
Zein	58.7 ± 1.5	61.5 ± 0.8	66.4 ± 2.8
Gelatin[2]	69.8 ± 1.5	67.8 ± 1.0	70.3 ± 3.0
Amino acid[2]	75.9 ± 1.6	70.4 ± 1.9	72.8 ± 1.7

[1] Standard error of the mean.
[2] Food intake in 30 minutes varied from 2.5 to 5.0 gm.

TABLE 2

Amount of insoluble nitrogen in intestinal contents

Diet	0-hours	1-hour	3-hours
	mg	*mg*	*mg*
"Nitrogen-free"	1.80 ± 0.13[1]	2.11 ± 0.25	2.62 ± 0.35
Amino acid	3.31 ± 0.44	2.16 ± 0.36	2.41 ± 0.42
Casein	2.51 ± 0.61	3.35 ± 0.63	4.05 ± 0.44
Zein	14.30 ± 1.10	26.47 ± 5.54	36.82 ± 3.61
Gelatin	2.57 ± 0.42	6.07 ± 1.30	4.27 ± 1.10

[1] Mean ± standard error.

The results shown in table 3 indicate that the TCA-precipitable nitrogen increased a little when the diet contained casein, zein or gelatin. The values were significantly ($P < 0.05$) higher than the corresponding values for rats fed the "nitrogen-free" diet at zero time and at one hour. They were also higher than those for rats fed the amino acid diet but only the three greatest differences, as indicated in table 3, were significant.

The values for TCA-soluble nitrogen (table 4) in the intestinal contents of rats fed the casein, zein or amino acid diets were between 2 and 4 mg higher than those for rats fed the "nitrogen-free" diet. A much greater accumulation of TCA-soluble nitrogen occurred in the intestinal contents of rats fed the diet containing gelatin. The differences between these values and the highest of those for rats fed the other diets were highly significant ($P < 0.001$) at each time interval.

The results of the final experiment are shown in table 5. The average values for the individual fractions were somewhat

TABLE 3

Amount of soluble nitrogen of intestinal contents that was trichloroacetic acid-precipitable

Diet	0-hours	1-hour	3-hours
	mg	*mg*	*mg*
"Nitrogen-free"	1.21 ± 0.08[1]	1.00 ± 0.07[1]	1.10 ± 0.10[1]
Amino acid	1.43 ± 0.11	1.13 ± 0.07	1.21 ± 0.12
Casein	1.63 ± 0.12	1.38 ± 0.12	1.74 ± 0.12[2]
Zein	1.66 ± 0.18	1.47 ± 0.18	1.46 ± 0.14
Gelatin	1.91 ± 0.10[2]	1.73 ± 0.15[2]	1.57 ± 0.13

[1] Mean ± standard error.
[2] Significantly different from corresponding values for amino acid group, at zero and 3 hours, ($P < 0.05$); at one hour, ($P < 0.01$).

TABLE 4

Trichloroacetic acid-soluble nitrogen of intestinal contents

Diet	0-hours	1-hour	3-hours
	mg	*mg*	*mg*
"Nitrogen-free"	3.52 ± 0.29[1]	2.47 ± 0.20	4.08 ± 0.53
Amino acid	7.24 ± 0.58	6.81 ± 0.70	7.44 ± 0.93
Casein	5.39 ± 0.60	6.10 ± 0.62	6.30 ± 0.44
Zein	6.71 ± 0.28	6.69 ± 0.57	7.39 ± 1.26
Gelatin	15.74 ± 1.03	19.45 ± 1.31	16.85 ± 0.84

[1] Mean ± standard error.

TABLE 5

Nature of the trichloroacetic acid-soluble nitrogen in the intestinal contents[1]

Diet	No. of rats	Insoluble[2] residual nitrogen	Trichloro-acetic acid-[2] precipitable nitrogen	Trichloroacetic acid-soluble fraction			
				Total[2] nitrogen	Amino[3] acid nitrogen	α-Amino[4] nitrogen	Estimated no. of amino acids/ peptide[5]
		mg	*mg*	*mg*	*mg*	*mg*	
Casein	5	1.78	0.59	4.80	2.00	3.03	2.7
Zein	5	15.50	0.66	8.93	3.93	5.33	3.5
Amino acid	5	1.65	0.64	7.53	4.75	5.77	2.8
"Nitrogen-free"	4	2.87	0.65	5.23	1.74	3.33	2.4
Gelatin	3	3.13	0.50	14.15	3.34	5.11	6.0

[1] Values are taken from a pooled sample of the number of rats indicated.
[2] Nitrogen determined by semimicro Kjeldahl.
[3] Nitrogen determined by ninhydrin method of Van Slyke et al. ('41).
[4] Nitrogen determined by formol titration.
[5] Calculated as follows: average peptide length = $\frac{\text{total nitrogen} - \text{amino acid nitrogen}}{\text{α-amino nitrogen} - \text{amino acid nitrogen}}$

lower in this experiment but the trends were similar to those of previous experiments. Although the total amount of TCA-soluble nitrogen was highest in the contents of the small intestine after feeding the gelatin diet, amino acid nitrogen and α-amino nitrogen were highest after feeding the amino acid diet. The last column of table 5 contains estimates of the average number of amino acid residues in the peptides, calculated from this information. The average number of residues in the nitrogenous compounds of the TCA-soluble fraction obtained after feeding the gelatin diet was estimated to be 6 compared with 2.5 to 3.0 for those obtained after feeding the other diets. These figures can be considered only as rough approximations because a preponderance of basic amino acids in the peptides would result in overestimation of the number of residues per peptide.

DISCUSSION

Stomach-emptying. Previously gelatin and amino acid diets were noted to empty from the stomach at nearly the same rate as diets containing other proteins (Rogers et al., '60); but in the present study the gelatin diet emptied more rapidly and the amino acid diet more slowly than the one containing casein. In these experiments, unlike the previous ones, the rats were not allowed to adapt to the gelatin and amino acid diets and the amounts consumed were less than 5 gm. The rate of emptying of a diet containing 15% of casein increases when the amount consumed is low (Peraino et al., '59), so the low intake of the gelatin diet may account for its rapid emptying. However, the amino acid diet, which was also consumed in smaller amounts, emptied very slowly after the first hour. Thus, the rate of emptying of this diet was not influenced in the same way by the quantity of food consumed, and the pattern of emptying apparently changed as the rats became adapted to it.

A large intake of a low-molecular-weight carbohydrate, which exerts a high osmotic pressure, depresses the food intake of rats fed a low-protein diet; also, the amount of moisture in the stomach contents is high shortly after the ingestion of such a diet (Harper and Spivey, '58). The stomach contents of rats fed the diets containing free amino acids or gelatin (which exert higher osmotic pressures than casein and zein) had high moisture contents and the food intakes of these groups were low. The effects were greater in unadapted rats than in rats allowed to adapt to the diets for a few days (unpublished results). This suggests that mechanisms controlling the amount of moisture in the stomach contents, as well as the rate of stomach emptying, are modified during the adaptation period in a way that enables the young rat to eat more of an amino acid diet.

Intestinal contents. The high value for nitrogen in the insoluble fraction (which probably represents largely undigested protein from the diet and nitrogen from mucosal cells sloughed off during the removal of the intestinal contents (table 2)) together with the average value for nitrogen in the TCA-soluble fraction (table 4), after feeding zein, suggests that its low digestibility is due rather to its insolubility than to peptide bonds that are resistant to proteolytic attack. This conclusion is supported by observations on the *in vitro* digestion of corn proteins (de Muelenaere et al., '61), indicating that the same pattern of peptides was released from native and "reconstituted" corn gluten.

If nitrogen in the TCA-precipitable fraction of the small intestinal contents of rats fed a protein-free diet or a diet containing only nitrogen from amino acids represents intact proteins from intestinal secretions, little of the additional nitrogen observed in the intestinal contents of rats fed diets containing protein would appear to arise from this source (table 3). The values for groups receiving protein were significantly higher at each interval ($P < 0.01$) than those for the group fed the protein-free diet but, in terms of total nitrogen ingested, the differences are small and suggest, in agreement with Geiger et al. ('58), that little nitrogen from this source accumulates in the intestine. Measurement at any one time gives no measure of the rate of turnover; hence this does not preclude the possibility suggested by Nasset ('57), that the quantity of nitrogen entering the intestine from secretions each day is large.

The amount of nitrogen in the TCA-soluble fraction of the intestinal contents was the same when the diet contained casein or zein as when it contained free amino acids. This, together with the observations that an amino acid diet empties from the stomach of rats adapted to such a diet at about the same rate as diets containing highly digestible proteins (Rogers et al., '60), indicates that the amino acids of a highly digestible protein such as casein are as readily available to the rat as are free amino acids.

The high values for nitrogen in the TCA-soluble fraction of the intestinal contents of rats fed gelatin are unexpected, because gelatin is known to be highly digestible (Mitchell and Block, '46). The low amino acid nitrogen (table 5) of the TCA-soluble fraction indicates that the amount of free amino acids observed after feeding gelatin was little or no greater than that after feeding the other diets. This suggests that gelatin or some peptides of gelatin are resistant to attack by the proteolytic enzymes of the rat. The low aromatic amino acid content of gelatin might well make it resistant to attack by pepsin and chymotrypsin. London and Rabinowitsch ('11) calculated that 94% of the nitrogen in chyme collected from a fistula of the lower stomach after feeding gelatin was peptide nitrogen, but only 86% was peptide nitrogen after feeding casein. There was very little difference between the percentages of peptide nitrogen after feeding these two proteins when the chyme was collected from the ileum. Geiger ('51) also observed that the amount of nitrogen in the intestinal contents of rats two hours after feeding was greater when the diet contained gelatin than when it contained casein.

In general, the results indicate that the nature of the dietary protein influences food intake, the passage of food from the stomach and the extent to which nitrogen accumulates in the intestine. The possibility that the rate of emptying of the stomach contents is influenced by the quantity of amino acids absorbed is suggested by the results of some of these experiments. When gelatin and zein diets were fed, nitrogen accumulated in the intestine owing to the slow or poor digestibility of these proteins; however, the rate of stomach-emptying was not delayed possibly because amino acids were being absorbed only slowly. On the other hand, the quantity of amino acids absorbed during the first hour after feeding the amino acid diet was quite high and the rate of stomach-emptying was delayed during the subsequent two hours.

SUMMARY

The amount of nitrogen in the stomach and intestinal contents of rats was determined at intervals after the animals had consumed a "nitrogen-free" meal or meals containing the equivalent of 22% of protein from casein, gelatin, zein or an amino acid mixture. When the meal contained free amino acids or gelatin, food intake was depressed, stomach-emptying pattern was altered and the moisture content of the stomach contents was increased compared with values for the casein control group.

More nitrogen was present in the intestinal contents of rats fed diets containing zein or gelatin than in those of rats fed casein or amino acids. The greatest part of the extra nitrogen was in an insoluble fraction when the diet contained zein and in a trichloroacetic acid-soluble fraction when the diet contained gelatin. Only small amounts of the nitrogen in the intestinal contents of rats fed the amino acid, casein, or "nitrogen-free" diets were in insoluble or trichloroacetic acid-precipitable fractions. The peptides from the intestinal contents of rats fed the gelatin diet were estimated to contain about twice as many amino acids as those from rats fed the other diets.

The amount of nitrogen in the soluble fraction of the intestinal contents of rats fed a diet containing protein or amino acids which was precipitable with trichloroacetic acid was small but was consistently greater than that observed when a "nitrogen-free" diet was fed.

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A Study of Experimental Hypercholesterolemia in the Mouse

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Hegsted et al. ('57a), recognizing the need for the quantitation of the effect of fats and oils on blood cholesterol, devised an assay in the rat made hypercholesterolemic by dietary cholesterol and cholic acid. In the present paper are presented some data obtained using a similar assay employing the weanling mouse as the experimental animal. Use of the mouse for such an assay offers certain advantages; conservation of materials in short supply, shorter time of assay, less space required and lower cost of animals. In addition, it yields desirable information in comparative biochemistry.

EXPERIMENTAL

Male weanling mice of the Sharp and Dohme strain were randomized in groups of 8, housed individually and allowed to feed ad libitum. The basal diet consisted of 20% of casein, 74% of glucose, 4% of salt mixture, 2% of cellulose and a vitamin addendum, which is described in detail in an earlier publication (Howe and Bosshardt, '60). In our experiments 1.0% of cholesterol and 0.1% of cholic acid were incorporated into the basal diet to produce a state of hypercholesterolemia. In later experiments 0.5% of linoleic acid was added to the basal diet as a source of essential fatty acids. It was reasoned that the effect of any substance on blood cholesterol might be influenced by the presence of a dietary deficiency. All dietary supplements were added at the expense of glucose.

After a 12-day period the animals were weighed and decapitated under light chloroform anesthesia to obtain pooled samples for plasma cholesterol determinations which were carried out by the method of Abell et al. ('52).

RESULTS AND CONCLUSIONS

The effects of a variety of dietary fats and fatty acids on plasma cholesterol are shown in table 1. In those experiments in which the diet contained 0.5% of linoleic acid the results given are the average of two separate experiments. Confirming our earlier observations (Howe et al., '60) linoleic acid and oils containing this fatty acid are ineffective in reversing cholic acid-induced hypercholesterolemia. Arachidonic acid also is inert in this respect. These observations contrast with those of Hegsted et al. ('57) in the rat, but there are experimental differences as well as a species difference. In these experiments only cod liver oil, a hexaenoic acid concentrate derived from cod liver oil, and tung oil at the highest level fed gave clear-cut reductions in plasma cholesterol. The effect observed with the highly unsaturated cod liver oil is not unexpected because marine oils have been reported to lower plasma cholesterol in both man (Bronte-Stewart et al., '56) and animals (Nimni et al., '61; Nicolaysen and Ragard, '61). The tung oil was effective only at a level that completely inhibited growth. This, too, is in contrast with the results of Hegsted, who found tung oil to be one of the most effective fats tested in elevating the plasma cholesterol of the rat.

In this investigation a hydrogenated coconut oil[1] and oleic acid were the only substances observed to potentiate the hypercholesterolemic effect of cholic acid and cholesterol in the mouse. The elevation due to palmitic acid failed to reach significance possibly because of poor absorption (Lin et al., '55).

Received for publication September 25, 1961.
[1] Hydrol, Durkee's Famous Foods, Chicago.

TABLE 1

Effect of fats on experimentally induced hypercholesterolemia in the mouse

Supplement[1]	No linoleic acid		0.5% Linoleic acid	
	Plasma[2] cholesterol	Weight gain	Plasma cholesterol	Weight gain
	mg/100 ml	*gm*	*mg/100 ml*	*gm*
None	200	9.0	180	11.2
Corn oil, 5%	170	11.0	175	11.3
Corn oil, 10%	191	10.9	164	11.4
Linseed oil, 5%	189	11.6	184	12.0
Linseed oil, 10%	189	11.3	190	11.8
Cod liver oil, 2%	144	10.3	157	11.4
Cod liver oil, 5%	140	11.4	151	11.1
Cod liver oil, 10%	152	11.4	160	11.1
Olive oil, 5%	210	11.1	204	11.5
Olive oil, 10%	192	10.5	214	11.6
Butter fat, 5%	217	11.4	216	11.2
Tung oil, 2%	205	8.2	223	8.5
Tung oil, 5%	182	2.3	202	3.6
Tung oil, 10%	154	−1.2	138	−0.2
Hydrogenated coconut oil,[3] 5%			248	12.6
Hydrogenated coconut oil,[3] 10%			242	12.7
Palmitic acid, 2%	170	11.2	222	12.3
Palmitic acid, 5%	222	10.3	201	14.2
Palmitic acid, 10%	224	6.1	227	12.7
Oleic acid, 5%	234	9.6	242	11.7
Oleic acid, 10%	262	8.8	252	10.2
Linoleic acid, 2.5%	163	10.2	186	11.1
Linoleic acid, 5%	192	8.8	198	11.1
Arachidonic acid concentrate,[4] 0.5%	184	9.2	170	11.0
Arachidonic acid concentrate, 1.0%	173	11.2	169	10.9
Arachidonic acid concentrate, 2.0%	191	9.5	172	10.6
Hexaenoic acid concentrate,[5] 0.5%	150	9.9	137	11.7
Hexaenoic acid concentrate, 1.0%	145	10.6	133	11.6
Hexaenoic acid concentrate, 2.0%	100	8.9	121	11.2

[1] All diets contain 1.0% of cholesterol and 0.1% of cholic acid. Plasma cholesterol of comparable mice fed basal diet was 101 mg/100 ml.
[2] Average standard deviation of each tabular value is 11.6.
[3] Hydrol, Durkee's Famous Foods, Chicago.
[4] A 60% concentrate supplied through the courtesy of Mr. R. Weston of Merck Sharp and Dohme Research Laboratories.
[5] A concentrate of cod liver oil consisting of approximately 67% of hexaenoic and 33% of pentaenoic acid. Supplied by Mr. F. Reimers of Merck Sharp and Dohme Research Laboratories.

TABLE 2

Effect of mixtures of hydrogenated coconut oil and safflower oil on experimentally induced hypercholesterolemia[1] in the mouse

Supplement	Plasma cholesterol	12-Day weight gain
Hydrogenated coconut oil,[2] 5%	258	14.1
Safflower oil, 5%	219	14.5
Hydrogenated coconut oil, 2.5% + safflower oil, 2.5%	202	14.4
Hydrogenated coconut oil, 10%	243	14.6
Safflower oil, 10%	214	14.4
Hydrogenated coconut oil, 5% + safflower oil, 5%	234	14.8

[1] All diets contained 1% of cholesterol and 0.1% of cholic acid.
[2] Hydrol.

TABLE 3

Effect of sex hormones on experimentally induced hypercholesterolemia in the mouse

Supplement[1,2]	Plasma[3] cholesterol	Weight gain
	mg/100 ml	*gm*
None	212	11.1
Diethylstilbestrol, 0.25 mg	219	8.2
Diethylstilbestrol, 0.5 mg	234	6.7
Diethylstilbestrol, 1.0 mg	208	5.5
Estradiol, 0.0625 mg	215	11.8
Estradiol, 0.125 mg	175	10.9
Estradiol, 0.25 mg	218	10.8
Estradiol, 0.5 mg	195	10.2
Estradiol, 1.0 mg	161	9.6
Progesterone, 12.5 mg	204	11.3
Progesterone, 25 mg	198	11.3
Progesterone, 50 mg	195	11.1
Testosterone, 12.5 mg	182	12.2
Testosterone, 25 mg	230	10.4
Testosterone, 50 mg	194	11.9
Testosterone, 100 mg	193	11.5

[1] All diets contained 0.5% of linoleic acid, 1% of cholesterol and 0.1% of cholic acid.
[2] All hormone supplements expressed in mg/kg of diet.
[3] Average standard deviation, 15.1.

TABLE 4

Effect of amino acids on experimentally induced hypercholesterolemia in the mouse

Supplement[1]	Series A Without added taurine		Series B With 1% taurine	
	Plasma[2] cholesterol	Weight gain	Plasma cholesterol	Weight gain
	mg/100 ml	*gm*	*mg/100 ml*	*gm*
None	206	11.4	159	13.3
DL-Alanine, 0.5%	184	11.5	175	13.1
DL-Alanine, 1.0%	191	10.7	176	11.6
L-Arginine·HCl, 0.5%	184	12.1	154	11.9
L-Arginine·HCl, 1.0%	190	10.7	150	11.7
L-Aspartic acid, 0.5%	210	11.1	152	12.2
L-Aspartic acid, 1.0%	170	11.1	202	12.6
L-Cystine, 0.5%	181	10.4	180	13.4
L-Cystine, 1.0%	166	10.8	167	12.5
L-Cysteine·HCl, 0.5%	155	11.0	185	12.9
L-Cysteine·HCl, 1.0%	163	10.1	168	10.1
Glycine, 0.5%	193	11.3	145	13.7
Glycine, 1.0%	197	10.7	193	12.1
Guanidoacetic acid, 0.5%	201	9.1	180	10.0
Guanidoacetic acid, 1.0%	183	5.4	195	7.3
L-Histidine HCl·H_2O, 0.5%	204	12.1	143	12.8
L-Histidine HCl·H_2O, 1.0%	191	9.4	183	12.1
L-Isoleucine, 0.5%, with alloisoleucine, 50%	185	11.7	146	12.5
L-Isoleucine, 1.0%, with alloisoleucine, 50%	199	10.8	175	13.0
L-Leucine, 0.5%	212	11.1	188	13.5
L-Leucine, 1.0%	177	11.6	161	12.5
L-Lysine·HCl, 0.5%	206	11.6	156	12.8
L-Lysine·HCl, 1.0%	205	11.6	168	12.2
DL-Methionine, 0.5%	173	11.3	121	11.8
DL-Methionine, 1.0%	173	10.4	130	9.7
DL-Phenylalanine, 0.5%	210	12.1	177	12.9
DL-Phenylalanine, 1.0%	222	12.1	188	13.7
L-Proline, 0.5%	208	12.6	157	12.4
L-Proline, 1.0%	207	11.4	186	13.1
DL-Serine, 0.5%	231	11.3	170	13.2
DL-Serine, 1.0%	222	11.6	159	12.7
DL-Threonine, 0.5%	250	11.2	139	13.5
DL-Threonine, 1.0%	202	11.6	164	13.4
DL-Tryptophan, 0.5%	225	11.8	149	12.3
DL-Tryptophan, 1.0%	192	11.1	185	12.7
L-Tyrosine, 0.5%	192	11.7	164	14.9
L-Tyrosine, 1.0%	201	11.4	162	15.0
DL-Valine, 0.5%	206	11.5	151	11.7
DL-Valine, 1.0%	185	12.2	126	12.8

[1] All diets contain 0.5% of linoleic acid, 1.0% of cholesterol and 0.1% of cholic acid.
[2] Average standard deviation, 22.1.

Since Hegsted and associates ('57b) had observed that in induced hypercholesterolemia the hypocholesterolemic effect of an oil appears to vary directly as the product of its saturated and essential fatty acid content (linoleic plus arachidonic), a study was made of combinations of safflower oil (70% linoleic) and hydrogenated coconut oil,[2] a highly saturated fat. The results are shown in table 2. In neither case did the combination of two fats produce a depression in blood cholesterol.

In table 3 are shown the data obtained in a study of the effect of sex hormones on cholic acid-induced hypercholesterolemia. Estradiol was the only substance found to produce a lowering effect and this effect was observed only at the highest concentration used. Diethylstilbestrol was without effect on plasma cholesterol even though all levels used caused greater growth inhibition than was observed at the highest concentration of estradiol.

Data obtained in the study of the effect of individual amino acids on induced hypercholesterolemia are shown in table 4, series A. Cystine and cysteine hydrochloride appeared to exert a hypocholesterolemic effect ($P < 0.05$ in analysis of variance of the data in individual tests). The effect obtained with methionine approached significance ($P = 0.1$). Portman and Mann ('55) have reported that taurine which is derived from cysteine reduces experimental hypercholesterolemia in monkeys. This effect was confirmed by Hermann ('59), in rats, but not in rabbits. The data in table 5 show that taurine is an effective agent in lowering plasma cholesterol in the mouse with experimental hypercholesterolemia.

The demand for taurine for conjugation of dietary cholic acid may exceed the capacity of the body to synthesize it. If such should be the case the resulting taurine deficiency might mask the effect of dietary amino acids. For this reason the amino acids were tested in the presence of taurine. The data are shown in table 4, series B. With taurine in the diet, cystine and cysteine caused no further reduction but the results obtained with methionine suggest that this amino acid may exert a plasma cholesterol-lowering effect even in the presence of taurine ($P = 0.1$).

TABLE 5

Effect of taurine on experimentally induced hypercholesterolemia in the mouse[1]

Supplement	Plasma cholesterol	12-Day weight gain
	mg/100 ml	*gm*
None	249	7.9
Taurine, 1%	169	7.8
Taurine, 2%	171	9.0
Taurine, 4%	235	8.8
None	269	8.4
Taurine, 0.1%	252	8.3
Taurine, 1.0%	200	7.8
None	312	8.0
Taurine, 1%	207	9.8
None	223	11.6
Taurine, 1%	182	9.3
Taurine, 4%	121	8.1
None	230	8.2
Taurine, 2%	157	9.8
Linoleic acid, 0.5%	214	12.4
Linoleic acid, 0.5%, taurine, 0.5%	149	10.9
Linoleic acid, 1.0%, taurine, 1.0%	143	14.3

[1] All diets contain 1% of cholesterol and 0.1% of cholic acid.

SUMMARY

In cholic acid-induced hypercholesterolemia in the mouse the following substances were observed to produce a plasma cholesterol-depressing effect: cod liver oil, hexaenoic acid concentrate, tung oil, estradiol, cystine, cysteine hydrochloride and taurine.

Tung oil was effective only at a dietary level that produced a growth inhibition.

Dietary taurine eliminated the hypocholesterolemic effect of cystine and cysteine hydrochloride.

Oleic acid and a hydrogenated coconut oil enhanced the hypercholesterolemic effect of cholic acid.

ACKNOWLEDGMENT

The authors are indebted to Maria Kryvokulsky and James L. Gilfillan for technical assistance throughout the course of this work.

[2] See footnote 1.

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Enhancing Effect of Aminonucleoside-Induced Proteinuria on the Dietary Choline Requirement of the Rat[1,2]

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Patients affected with proteinuria of the nephrotic syndrome, especially children, are usually undernourished and some may have fatty livers. Urinary proteins of the nephrotic child have been observed in one series of Albanese et al. ('49) to contain an average of 6.3 gm of methionine per 100 gm of protein. Rapidly developing and extensive fatty change in the liver is produced in rats (Best and Huntsman, '32), pigs (Johnson and James, '48), dogs (Burns and McKibbin, '51), calves (Johnson et al., '51), mice (Wilson, '51), guinea pigs (Casselman and Williams, '54), rabbits (Hove et al., '54), chickens (Salmon and Copeland, '54), and cats (Carvalho Da Silva et al., '59) fed a choline-deficient diet. In this paper, studies have been made concerning the effect of an experimental nephrotic syndrome on dietary choline requirements in the rat.

Aminonucleoside, (6-dimethyl-amino-purine-3-amino-D-ribose) was utilized by Frenk et al. ('55) in inducing a nephrotic syndrome in rats by subcutaneous injection. We used this method to provoke proteinuria by oral feeding of aminonucleoside (0.015%) incorporated in the diet (Borowsky et al., '61). The lipotropic requirements of the animals under these conditions were estimated by measuring the amount of fat in the liver by biochemical methods (Payne, '49).

MATERIALS AND METHODS

Sixty male Wistar albino rats (90 to 100 gm) were allocated to 4 groups and housed in individual cages with wire bottoms (to limit coprophagia), in a temperature-controlled environment. Urine was collected over 24-hour periods from each rat for protein determination (Ham, '50). Levels of total and fractional serum proteins (Lowry et al., '51), and serum cholesterol (Saifer and Kammerer, '46) were likewise estimated. The rats were then fed the following diets:

Group 1	Choline-deficient diet with aminonucleoside
Group 2	Choline-deficient diet alone
Group 3	Choline-supplemented diet with aminonucleoside
Group 4	Choline-supplemented diet alone

The basal diet was choline-deficient (table 1). Either choline chloride (1.0%) or aminonucleoside (0.015%), or both, were added at the expense of sucrose. This relatively high level of choline was used to insure that even under abnormal conditions, sufficient would still be available to the animal. Dry ingredients of the diets were mixed thoroughly, and the supplements added and blended before the addition of fat. The food was stored in sealed plastic containers at 4°C to prevent rancidity. Rats in group 1 were offered the diet ad libitum, the amount of food eaten recorded daily and the uneaten portions discarded after weighing. Corresponding rats in groups 2, 3 and 4 started to receive their diets a day or two later. They were offered and they consumed the same amount of food that the corresponding rat in group 1 had eaten the day before. Urine over a 24-hour period was collected once weekly from each rat for the first two weeks, and daily thereafter. If a rat died in group 1,

Received for publication September 11, 1961.

[1] Presented in part before the Federation of American Societies for Experimental Biology.

[2] This investigation was supported in part by U.S.P.H.S. Grant no. C-2548.

TABLE 1

Basal diet (choline-deficient)

	%
Casein, vitamin-free	5.0
Alpha soya protein, vitamin-free	5.0
Soybean protein[1]	7.0
Cellulose	2.0
Sucrose	42.9
Inorganic salt and trace element mixture[2]	4.0
Vitamin mixture without choline and B_{12}[3]	3.0
Cystine	0.1
Hydrogenated cottonseed oil[4]	29.0
Corn oil	2.0

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

[2] Composition in per cent: calcium carbonate, 21.000; copper sulfate (5 H_2O), 0.039; ferric phosphate, 1.470; manganous sulfate (anhydrous), 0.020; magnesium sulfate (anhydrous), 9.000; potassium aluminum sulfate, 0.009; potassium chloride, 12.000; potassium dihydrogen phosphate, 31.000; potassium iodide, 0.005; sodium chloride, 10.500; sodium fluoride, 0.057; tricalcium phosphate, 14.900.

[3] Each gram of the vitamin mixture contained in milligrams: thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; niacin, 4.5; folic acid, 0.09; menadione, 2.25; *p*-aminobenzoic acid, 5.0; ascorbic acid, 45.0; also vitamin A, 900 IU; vitamin D, 100 IU; biotin, 20 μg.

[4] Crisco, Procter and Gamble Company, Cincinnati.

which happened more often than in the other groups, an autopsy was done, organs weighed, and the kidney-body weight and liver-body weight ratios computed and expressed in per cent. Hepatic lipid concentrations (wet-weight) were determined on fresh tissues by chloroform extraction. Blocks of tissues from the liver and kidneys were fixed in Bouin's solution and cobalt-formol; frozen sections were cut from the block fixed in cobalt-formol, and stained for fat with oil red O (Wilson, '50). Paraffin sections from tissues fixed in Bouin's solution were stained with hematoxylin and eosin. The corresponding rats in groups 2, 3, and 4 were killed and subjected to the same procedure after comparable periods on their respective dietary regimens. The rats that survived were watched closely and as soon as any in group 1 developed a significant degree of proteinuria, they were killed, and total protein, serum albumin and serum cholesterol determined from the blood collected at the time of decapitation. Autopsies were done, organs weighed, and hepatic lipid concentrations determined. The corresponding rats in groups 2, 3, and 4 were killed at comparable periods and subjected to the same procedure.

It was considered pertinent to this study to determine the incidence of fatty liver in nephrotic patients. Thirty-five consecutive autopsies of patients dying with subacute glomerulonephritis (with recorded proteinuria) were collected from the files of the Department of Pathology of Barnes Hospital as well as 35 other cases selected as controls of comparable age and sex (but free of glomerulonephritis).

RESULTS

Food intake. The average daily food intake was about 8 gm.

Increase in weight. Body weights of rats in groups (3 and 4) fed choline-supplemented diets increased progressively, whereas those in groups (1 and 2) without choline - supplements initially increased, reaching a peak from the eighth to the eleventh day, but decreased later (fig. 2). The weights after the seventeenth day were not represented because the remaining animals were killed from the seventeenth to the twenty-first day.

Mortality. After the rats had received their diets for 12 to 17 days, 8 animals died with hemorrhagic kidneys in group 1; two in group 2 and none in either of the choline-supplemented groups (table 2). No other rats died of hemorrhagic kidneys thereafter.

Proteinuria. Abnormal amounts of protein were not detected in the urine of any rats at the start of the experiment. Definite proteinuria developed in rats of group 1 (choline-deficient with aminonucleoside) after being fed the diet for 17 to 21 days and the amount of protein measured was significantly more than for group 3 (choline-supplemented with aminonucleoside); 95.7 mg per day vs. 34 mg per day ($P < 0.001$). Proteinuria did not develop in those groups (2 and 4) that did not receive aminonucleoside (table 2).

Hepatic histology and lipid concentration. Numerous lipid vacuoles were noted in the parenchyma of the centrolobular zones in both choline-deficient groups (fig. 3), but none in the choline-supplemented ones. Hepatic lipid determinations for the choline-supplemented groups were within

TABLE 2

Summary of data (mean values)

	Days fed diet	Choline-deficient group		Choline-supplemented group	
		1 (with amino-nucleoside)	2 (without amino-nucleoside)	3 (with amino-nucleoside)	4 (without amino-nucleoside)
Proteinuria, mg/24 hrs.	17–21	95.71 ± 6.28[1]	1.60 ± 1.43[1]	34.00 ± 10.41[1]	0.14 ± 0.12[1]
Hepatic lipid concentration, %	17–21	20.16 ± 2.26	12.24 ± 2.52	4.32 ± 0.19	5.28 ± 1.97
Hepatic histology	12–21	centrolobular fat	centrolobular fat	no fatty change	no fatty change
Renal histology	12–21	numerous casts in tubules	no casts in tubules	few casts in tubules	no casts in tubules
Blood total protein (gm/100 ml change from initial values)	17–21	−1.60 ± 0.38	normal +0.64	−0.70 ± 0.31	normal −0.38
Liver-body weight ratio, %	12–21	7.63 ± 0.32	5.45 ± 0.45	3.78 ± 0.14	3.86 ± 0.21
Kidney-body weight ratio, %	12–21	2.0 ± 0.1	1.5 ± 0.1	1.2 ± 0.05	1.1 ± 0.06
Serum albumin, gm/100 ml	17–21	1.48 ± 0.14	normal 3.50	1.88 ± 0.15	normal 2.68
Serum cholesterol, mg/100 ml	17–21	250.6	94.0	260.0	120.3
Mortality	12–17	8	2	0	0
Weight	1–21	increased then decreased	increased then decreased	progressive increase	progressive increase

[1] Standard error of the mean.

normal limits, whereas those in both choline-deficient groups were elevated (table 2). Significant difference was found between the hepatic lipid concentrations in groups 1 (choline-deficient with aminonucleoside) and 2 (choline-deficient alone), higher values being obtained in group 1.

Renal histology. In sections of the kidneys from group 1 (choline-deficient with aminonucleoside), numerous hyaline casts were present in tubules, proximal portions being most severely affected (fig. 4). Kidneys of rats in those groups (2 and 4) that did not receive aminonculeoside were free of tubular casts and hemorrhagic changes. That group (3), fed the choline-supplemented diet with aminonucleoside, showed only few casts in tubules of the kidneys.

Blood total protein. The values at the time the animals were killed, in those groups (2 and 4) not fed with aminonucleoside, did not show any significant change from initial ones. Group 1 (choline-deficient with aminonucleoside) showed a significant diminution (−1.6 gm/100 ml) as compared with all the other groups (table 2). The values in group 3 (choline-supplemented with aminonucleoside) were not significantly different from those of groups 2 and 4.

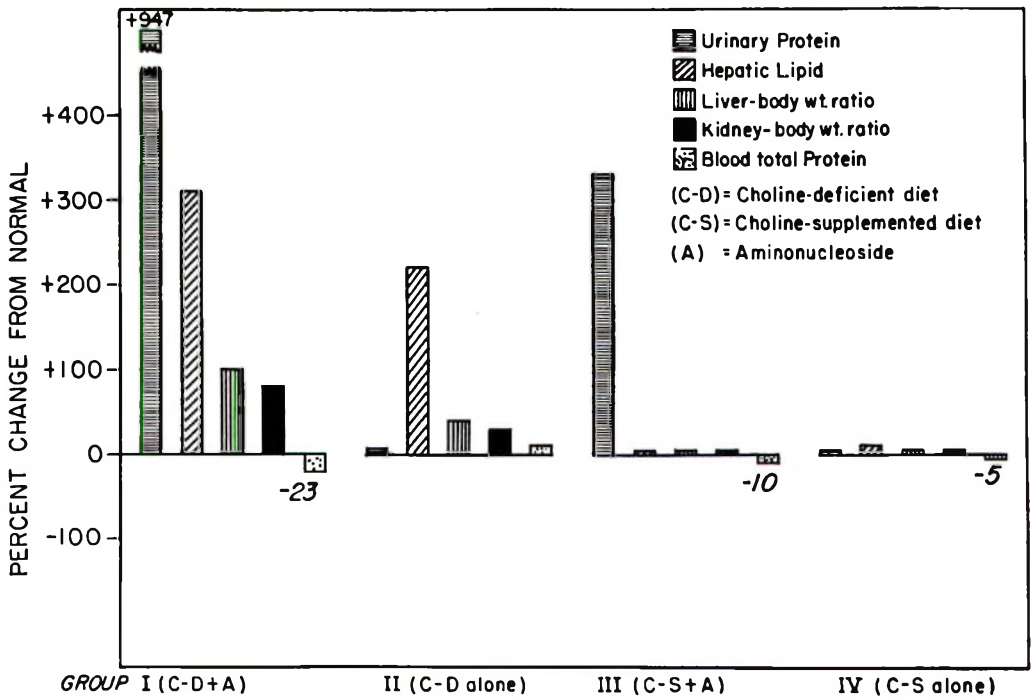

Fig. 1 Mean values of urinary protein, hepatic lipid, liver and kidney-body weight ratios, and blood total protein after rats were fed diet 17 to 21 days.

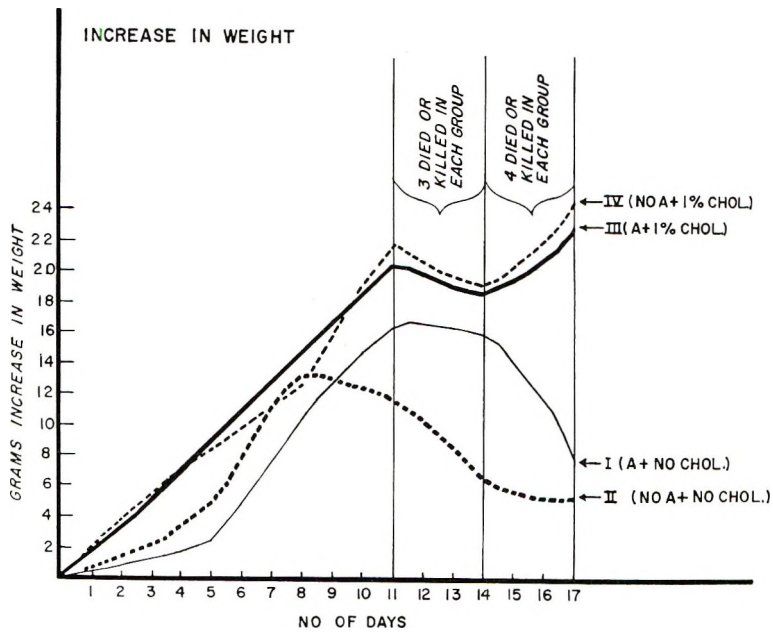

Fig. 2 Grams increase in weight.

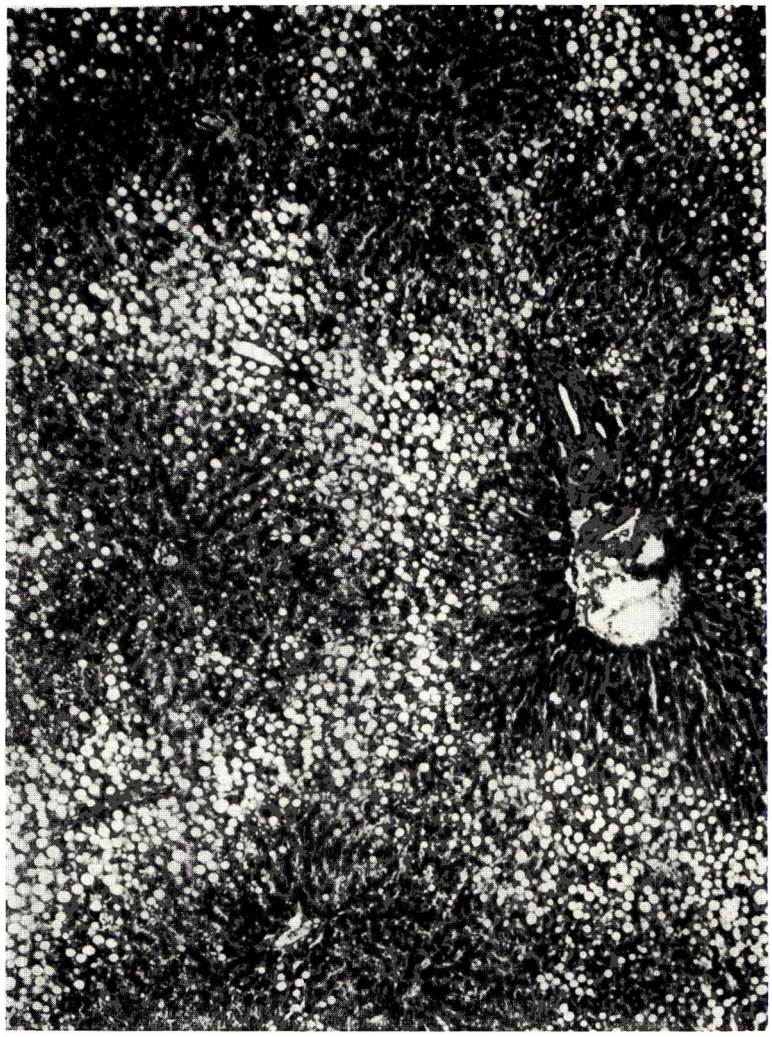

Fig. 3 Low power photomicrograph of the liver of rat fed the choline-deficient diet showing fatty change in the centrolobular zones. H & E.

Kidney - body weight and liver - body weight ratios. Those rats fed the diet supplemented with choline chloride (groups 3 and 4) did not show any increase in the kidney-body weight ratios, whereas the ratios of those fed the choline-deficient diets (groups 1 and 2) increased. The values were highest in that group fed aminonucleoside in the choline-deficient diet above. The observations in the liver-body weight ratios parallel those of the kidney-body weight ratios (table 2).

Serum albumin. There was a significant diminution in the values for both groups given aminonucleoside, but the administration of choline (group 3) did not prevent this fall in serum albumin. Those not fed with aminonucleoside did not have hypoalbuminemia (table 2).

Serum cholesterol. Serum cholesterol for groups fed aminonucleoside showed significantly higher values than those of the other groups (table 2). There was also no significant difference between the values for the aminonucleoside-treated groups whether fed the basal diet or the latter supplemented with choline. Groups not

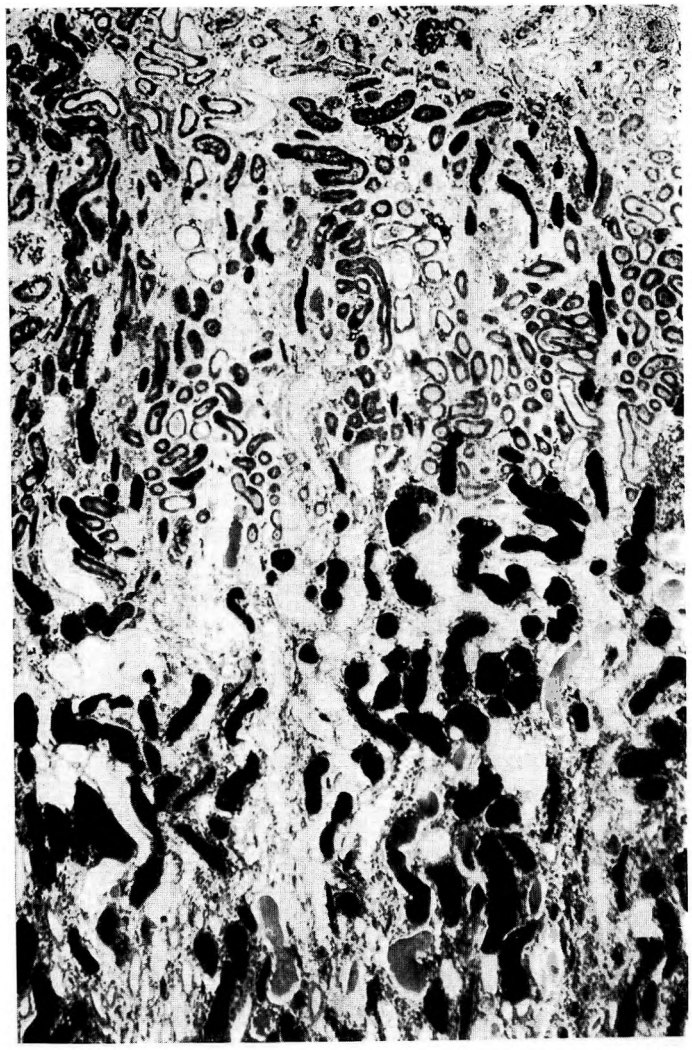

Fig. 4 Low power photomicrograph of the kidney of rat in group 1 (choline-deficient with aminonucleoside) showing the casts in the tubules. PAS.

fed with aminonucleoside did not show hypercholesterolemia.

The mean values (per cent change from normal) of the urinary protein, hepatic lipid concentrations, liver and kidney-body weight ratios and total blood protein in the different groups are illustrated in figure 1. The values in group 4 are all within normal limits. The values in group 1 are all significantly different from all the other groups. The urinary protein and blood total protein in group 2 are within normal limits. The liver and kidney-body weight ratios are significantly higher in groups 1 and 2 than those of groups 3 and 4. All the values in group 3 are within normal limits except the urinary protein.

Human material. Of the 35 cases of subacute glomerulonephritis, 10 (28%) were found to have fatty change in the liver. Of the 35 control cases of comparable age, only 2 (6%) were found to have fatty change in the liver.

DISCUSSION

A mild degree of nephrotic syndrome was produced in rats fed aminonucleoside for 17 to 21 days, as evidenced by pro-

teinuria, hypoproteinemia and hypercholesterolemia. Flood ('50) has strongly suggested that methylation in nephrotics is subnormal. Albanese et al. ('49) have reported the presence of considerable amounts of methionine in urinary proteins excreted by a nephrotic child. In our experiments the choline - deficient group that received aminonucleoside (group 1) lost considerable protein in the urine, as compared with group 2 (choline-deficient alone) which did not develop any proteinuria. The group fed the choline-deficient diet alone (group 2) developed fatty liver. but the hepatic-lipid concentration was significantly less than in the group that developed proteinuria (group 1: choline-deficient diet with aminonucleoside). These observations suggest that loss of methionine in the urine occurred in group 1, to deprive the system of some of the available preformed methyl groups necessary for the transmethylation reaction in the synthesis of choline. The deprivation would diminish the amount of lipotropic factors available to the body thereby increasing the oral requirement as evidenced by a corresponding increase in hepatic lipid concentrations. In the group fed the choline-supplemented diet with aminonucleoside (group 3), less protein was lost in the urine than in group 1. The hepatic lipid concentration in this group (group 3) was within normal limits since the diet contained a high supplement of choline chloride. The amount of choline added to this diet and that fed rats of group 4 was deliberately large (1.0%) to insure that under these conditions the controls would receive completely adequate amounts.

The lobular distribution of the fatty change in the liver was centrolobular. The localization conforms with that observed in choline deficiency (Hartroft, '50). Sidransky and Farber ('58) have shown that methionine-deficient diets induced fatty livers (periportal) in adult female rats, but not in adult males. We believe that the amount of methionine excreted in the urine of nephrotic rats fed the choline-deficient diet is not sufficient to produce changes of methionine deficiency, but rather the amount of methyl group available for the formation of choline is diminished, thus aggravating the existing choline deficiency as evidenced by the increase in hepatic lipid concentration. In any case methionine deficiency has been reported to produce periportal fatty liver only in female rats; male rats were used in our study. Because rats of all 4 groups received a supplement of cysteine (0.1%), a deficiency of this amino acid is not likely involved. Again, the centrolobular distribution of fat in livers of rats in group 1 would not support the concept of this amino acid deficiency which leads to periportal fatty livers.

It would appear from our animal experiments described, that under these conditions proteinuria increased the relative requirement of dietary lipotropic agents because of the loss of methionine in the urine thereby decreasing availability of labile methyl groups for *in vivo* synthesis of choline. The indirect evidence afforded by the autopsy data would support the hypothesis that a similar situation may exist in nephrotic children, at least those who had developed fatty livers. Because of these observations, the possibility arises that in selected cases of severe proteinuria in children (where protein requirements are high in any case because of growth demands) administration of pharmaceutical lipotropic preparations should be considered.

Ridout et al. ('54) discovered that dietary choline enhances the elevation of cholesterol in the serum when supplements of cholesterol (0.8%) are added to the diets of rats. No such effect was evident in our experiments when serum cholesterol was elevated endogenously (groups 1 and 3; table 2). Our data do not explain this discrepancy of the cholesterogenic effect of choline on the serum in these two situations.

SUMMARY

Fatty livers were produced in rats by feeding a choline-deficient diet (groups 1 and 2). Concomitant proteinuria was produced in one group (1) by adding aminonucleoside in the diet. Hepatic lipid concentrations in the group fed a choline-deficient diet with aminonucleoside (1) were significantly higher than those in the

group fed the choline-deficient diet alone (2). These data suggest that an increase in the exogenous lipotropic requirement was induced by the loss of methionine-containing protein in the urine of the nephrotic animals fed the choline-deficient diet.

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Dog Serum Lipid Responses to Dietary Fats Differing in the Chain Length of the Saturated Fatty Acids[1]

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Controlled dietary experiments in man have shown that the glycerides of saturated fatty acids with 12- and 14-carbon atoms cause slightly higher serum cholesterol levels than equal weights of glycerides containing saturated fatty acids of 16- and 18-carbon atoms (Grande et al., '61).

It has been reported that glycerides containing mainly saturated fatty acids of 8- and 10-carbon atoms do not increase serum cholesterol concentration in man when added to a low-fat diet (Beveridge et al., '59) and that they produce serum cholesterol levels only slightly higher than corn oil and lower than butterfat (Hashim et al., '60).

The observation in this laboratory that coconut oil, when added to a low-fat diet, produces a considerable elevation of the serum cholesterol level in the dog, gave an opportunity to compare in this animal the effects on the serum lipids of dietary fats differing in the chain length of their saturated fatty acids.

The experiments to be reported offer a direct comparison of the effects on the serum lipids of the dog of equal amounts of glycerides of similar composition in terms of saturated, monoene, and polyene fatty acids, but differing in the chain length of a part of the saturated fatty acids. Two such comparisons were made: one (exp. 27 and 28) between coconut oil and a glyceride containing mainly saturated fatty acids of 8- and 10-carbon atoms (MCT); the other (exp. 30) between a mixture of coconut oil and olive oil containing saturated fatty acids mainly of 12- and 14-carbon atoms, and a mixture of oleostock and totally hydrogenated corn oil containing saturated fatty acids mainly of 16 and 18 carbons. In all the comparisons the amount of experimental fat corresponded approximately to 40% of the total caloric intake.

The results showed that the glycerides of saturated fatty acids with 12 and 14 carbons produced a greater elevation of serum cholesterol than either the glycerides of 16- and 18-carbon saturated fatty acids, or of 8- and 10-carbon saturated fatty acids. The glycerides of the 8- and 10-carbon saturated fatty acids produced serum cholesterol levels only slightly higher than those observed with a low-fat diet with 4% of fat calories.

EXPERIMENTAL

Male adult mongrel dogs, kept in the laboratory, were fed a constant low-fat, low-cholesterol diet for more than one month before use in the experiments. All experiments followed a reversal design. In each instance the animals were divided into two equal groups matched as to body weight and serum cholesterol level, in the low-fat diet. After the dogs were fed the standard low-fat diet for several weeks, each of the two groups was fed one of the two diets to be compared for a period of two weeks. The diets were then exchanged and given for another two weeks. Finally, all the animals were again fed the standard low-fat diet.

Blood samples for lipid analysis were taken twice at the end of the control low-fat period (control 1), at the end of the first week of each experimental period and twice at the end of the second week of each experimental period. Blood samples were

Received for publication October 16, 1961.

[1] Supported by grants of the John A. Hartford Foundation, New York, and the Mount Sinai Hospital Research Fund.

taken in the same way during the second low-fat period (control 2). Body weights were determined weekly throughout the experiment.

Diets. The basic low-fat diet was made by mixing 95 parts by weight of a commercial dog food,[2] which by analysis had a fat content of 1.6% (corresponding approximately to 4% of the total calorie value), and 5 parts of nonfat dry milk solids. Ten milliliters of cod liver oil were added per kilogram of dog food. The amount of digitonin precipitable, Liebermann-Burchard reacting material in the commercial dog food was equivalent to 18 mg of cholesterol per 100 gm. The experimental diets were prepared by mixing carefully by weight, 80 parts of the basic low-fat diet and 20 parts of the experimental fat. During the experimental periods a weighed amount of diet was placed in the feeders each morning, and the daily food intake for each dog was measured by subtracting the weight of the food left in the feeder 24 hours later.

Experiment 27 was a direct comparison between coconut oil and a synthetic medium-chain triglyceride (MCT)[3] containing glycerides of saturated fatty acids mainly of 8- and 10-carbon atoms. The dogs ate the diets poorly, especially the MCT diet, and lost weight during the experiment. For this reason, a new experiment (exp. 28) was designed.

In experiment 28, the experimental fats were given by stomach tube in the form of a 15.15% emulsion in skim milk containing 5% of nonfat dry milk solids. The emulsion was prepared daily with a Waring Blendor. Twenty-two milliliters of this emulsion per kilogram were given daily. This corresponds to 3.33 gm of fat per kg, or 40% of the total calorie intake assuming a mean calorie intake of 75 Cal. per kg per day. The dogs were also given the commercial dog food (with 1% of cod-liver oil) and the amount eaten was measured daily as indicated. The total calorie intake was computed for each dog from the amount of fat emulsion given by stomach tube and the amount of commercial dog food eaten.

To match the content of total saturated, total monoene and total polyene fatty acids of the two experimental fats, olive oil, and safflower oil were added to MCT in this experiment. The resulting MCT mixture consisted of 92 parts of MCT, 6 parts of olive oil and two parts of safflower oil by weight.

Experiment 30 was a comparison of two fat mixtures, one containing saturated fatty acids mainly of 12- and 14-carbon atoms (12C) and the other containing saturated fatty acids mainly of 16- and 18-carbon atoms (18C), in the presence of monoene and polyene fatty acids in equal proportions in the two mixtures. Mixture 12C consisted of 60 parts of coconut oil and 40 parts of olive oil by weight. Mixture 18C consisted of 80 parts by weight of oleostock and 20 parts of totally hydrogenated corn oil. Oleostock is the filtered fat from beef internal organs with some admixture of fat trimmings from the carcass (see Grande et al., '61). The fats were given in this experiment mixed with the basic low-fat diet as in experiment 27.

The fatty acid composition of the experimental fats was established by gas-liquid chromatography analysis. Two independent sets of analyses were carried out on each sample, one in the Laboratory of Physiological Hygiene and another in the Jay Phillips Research Laboratory, using, respectively, a Beckman model GC-2 apparatus and an Aerograph model A 110-C. Columns of Craig's butanediol succinic acid polyester were used in each analysis. The analytical results are presented in table 1.

The differences between the two fats used in each experiment, in terms of fatty acids, are reported in table 2. The column headed "Excess" expresses the net differences in grams between 100-gm portions of the two experimental fat supplements used in each experiment, for each individual fatty acid. These differences were computed using the analytical values reported in table 1.

Analytical methods. Total serum cholesterol was determined by the method of Abell et al. ('52), lipid phosphorus by the method of Youngburg and Youngburg ('30) and total esterified fatty acids by the

[2] Kibbies, Morton Dog Food Company, Minneapolis 4, Minnesota.

[3] Kindly supplied by Dr. V. C. Babayan of the E. F. Drew Co., Inc., Boonton, New Jersey.

TABLE 1

Fatty acid composition (as percentage of the total fatty acids) of the oils and fat mixtures used in experiments 27, 28 and 30

Fatty acids[1]	Coconut oil	MCT	MCT[2] mixture	12C[3] mixture	18C[4] mixture
Saturated					
6:0	1.6	0.8	0.7	1.1	3.5
8:0	8.0	67.2	61.8	4.7	0
10:0	6.7	31.5	29.0	4.1	0.1
12:0	49.0	0.5	0.5	30.5	0.2
14:0	18.1	0	0.3	11.5	4.6
16:0	8.2	0	1.1	9.9	28.0
18:0	2.0	0	0.1	2.0	31.1
Monoene					
14:1	0	0	0	0	1.3
16:1	0	0	0.1	0.6	3.0
18:1	5.2	0	4.4	32.8	26.2
Polyene					
16:2	0	0	0	0	0.8
18:2	1.2	0	2.0	2.8	1.2
Total saturated	93.6	100.0	93.5	63.8	67.5
Total monoene	5.2	0	4.5	33.4	30.5
Total polyene	1.2	0	2.0	2.8	2.0

[1] The first figure represents the number of carbon atoms, the second the number of double bonds.
[2] MCT mixture: 92 parts of MCT, 6 parts of olive oil, 2 parts of safflower oil, by weight.
[3] 12C mixture: 60 parts of coconut oil and 40 parts of olive oil, by weight.
[4] 18C mixture: 80 parts of oleostock and 20 parts of totally hydrogenated corn oil, by weight.

TABLE 2

Comparison of fat supplements used in experiments 27, 28 and 30[1]

Fatty acids[2]	Experiment 27		Experiment 28		Experiment 30	
	Excess in coconut oil	Excess in MCT	Excess in coconut oil	Excess in MCT mixture	Excess in 12C mixture	Excess in 18C mixture
Saturated						
6:0	0.8	0	0.9	0	0	2.4
8:0	0	59.2	0	53.8	4.7	0
10:0	0	24.8	0	22.3	4.0	0
12:0	48.5	0	48.5	0	30.3	0
14:0	18.1	0	17.8	0	6.9	0
16:0	8.2	0	7.1	0	0	18.1
18:0	2.0	0	1.9	0	0	29.1
Monoene						
14:1	0	0	0	0	0	1.3
16:1	0	0	0	0.1	0	2.4
18:1	5.2	0	0.8	0	6.6	0
Polyene						
16:2	0	0	0	0	0	0.8
18:2	1.2	0	0	0.8	1.6	0
Total saturated	0	6.4	0.1	0	0	3.7
Total monoene	5.2	0	0.7	0	2.9	0
Total polyene	1.2	0	0	0.8	0.8	0

[1] The net differences in grams between 100-gm fractions of the two supplements used in each experiment are given as "excess" for each individual fatty acid.
[2] The first figure represents the number of carbon atoms, the second the number of double bonds.

method of Bauer and Hirsch ('49). The method of Bauer and Hirsch used in experiments 27 and 28 frequently gave values too low to account for the fatty acids of the cholesterol esters and the phospholipids. For this reason the method of Albrink ('59) was used for the determination of the total fatty acids in experiment 30. Triglycerides were computed assuming that one milliatom weight of P corresponds to 2 mEq of fatty acid and that 76% of the cholesterol is esterified. Milliequivalents of fatty acids were transformed into weights of triglycerides using the factor 285.7.

The fatty acid content of the stools was determined by the method of Van de Kamer ('58). The digitonin precipitable, Liebermann - Burchard reacting material of the stools was determined using an aliquot of the petroleum ether extract of the Van de Kamer method.

RESULTS

The first experiment (exp. 27) provides a comparison of the effects of coconut oil and of MCT on serum cholesterol. Eight dogs were used, but one was dropped from the experiment because of very poor eating. Data for the other 7 dogs are summarized in table 3.

Coconut oil produced higher serum cholesterol than MCT. The mean cholesterol difference, coconut oil minus MCT, was 80 mg per 100 ml (SE of the mean ± 25.0) with a probability of chance occurrence of $P = 0.018$. Compared with the low-fat diet, MCT produced an average elevation of serum cholesterol concentration of 47 mg per 100 ml (SE of the mean ± 22.9) which was not statistically significant ($P = 0.09$). The average food consumption of the 7 dogs for the coconut oil periods was 59 Cal. per kg per day, and for the MCT periods 34 Cal. per kg per day. The 7 dogs had a mean body weight of 15.6 kg at the end of control 1; they showed a mean weight loss of 0.16 kg for the two weeks of the coconut oil periods and of 1.00 kg for the two weeks of the MCT periods. All the animals gained weight on returning to the low-fat diet (control 2).

TABLE 3

Total serum cholesterol in experiment 27[1]

Diets	Serum cholesterol	
	Mean	SE mean
	mg/100 ml	
Low-fat (control 1)[2]	170	±13.7
Coconut oil, 20%	310	±45.0
MCT, 20%	230	±27.3
Low-fat (control 2)[3]	195	±25.3
Cholesterol difference		
Coconut oil *minus* MCT	80	±25.0
	$P = 0.018$	

[1] Values are means and standard errors of the means for 7 dogs.
[2] Control 1, values for the low-fat diet before administration of the experimental fats.
[3] Control 2, values at the end of two weeks of feeding low-fat diet after administration of the experimental fats.

In experiment 28 the comparison between coconut oil and MCT was repeated on 12 dogs, but the experimental fats were given by stomach tube and MCT mixture was used in place of MCT, to match the monoene and diene content of coconut oil. The mean food intake of the 12 dogs was 77 Cal. per kg per day for the coconut oil periods and 70 Cal. per kg per day for the MCT mixture periods. Body weight was constant throughout. The mean serum cholesterol changes are shown in figure 1.

A marked increase of serum cholesterol was shown by the dogs receiving the coconut oil, whereas very little change was observed in the dogs receiving the MCT mixture. Upon exchanging the experimental fats, coconut oil produced an elevation of the cholesterol levels in the animals previously receiving MCT mixture, while administration of the latter to the dogs previously receiving coconut oil caused a marked decrease of their cholesterol level. The cholesterol levels returned to the control values after the dogs were fed the low-fat diet for two weeks. Similar changes were observed in the serum phospholipids and total esterified fatty acids. The analysis of the serum lipid change is presented in table 4. Coconut oil produced significantly higher serum cholesterol, phospholipids and total esterified fatty acids than the MCT mixture.

In experiment 30 the comparison of the two fat mixtures 12C and 18C was made. The mean serum cholesterol changes in this experiment are depicted in figure 2. Both the 12C and the 18C mixtures caused the cholesterol concentration to increase

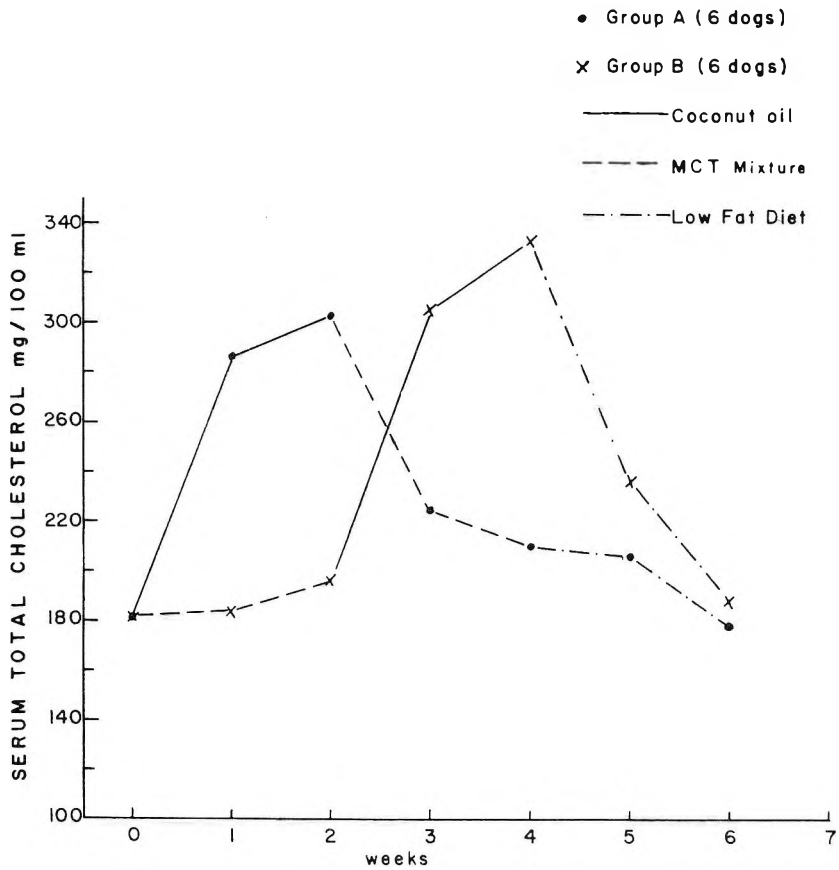

Fig. 1 The changes of serum total cholesterol in experiment 28. Values are means for the 6 dogs of each group.

TABLE 4

The serum lipids in experiment 28

	Diets			Difference coconut oil *minus* MCT	*P*
	Low-fat (control 1)	Coconut oil	MCT mixture		
Total cholesterol, mg/100 ml	181 ± 13.9	319 ± 22.9	203 ± 14.6	116 ± 15.1	< 0.0001
Phospholipids, mg/100 ml[2]	329 ± 20.3	505 ± 32.3	378 ± 21.2	127 ± 24.6	0.0004
Total esterified fatty acids, mEq[3]	9.4 ± 0.61	13.8 ± 1.13	9.3 ± 0.62	4.5 ± 0.95	0.0007

[1] Values are means and standard errors of the means for 12 dogs.
[2] Phospholipids = lipid phosphorus × 25.
[3] Total esterified fatty acids determined by the method of Bauer and Hirsch ('49).

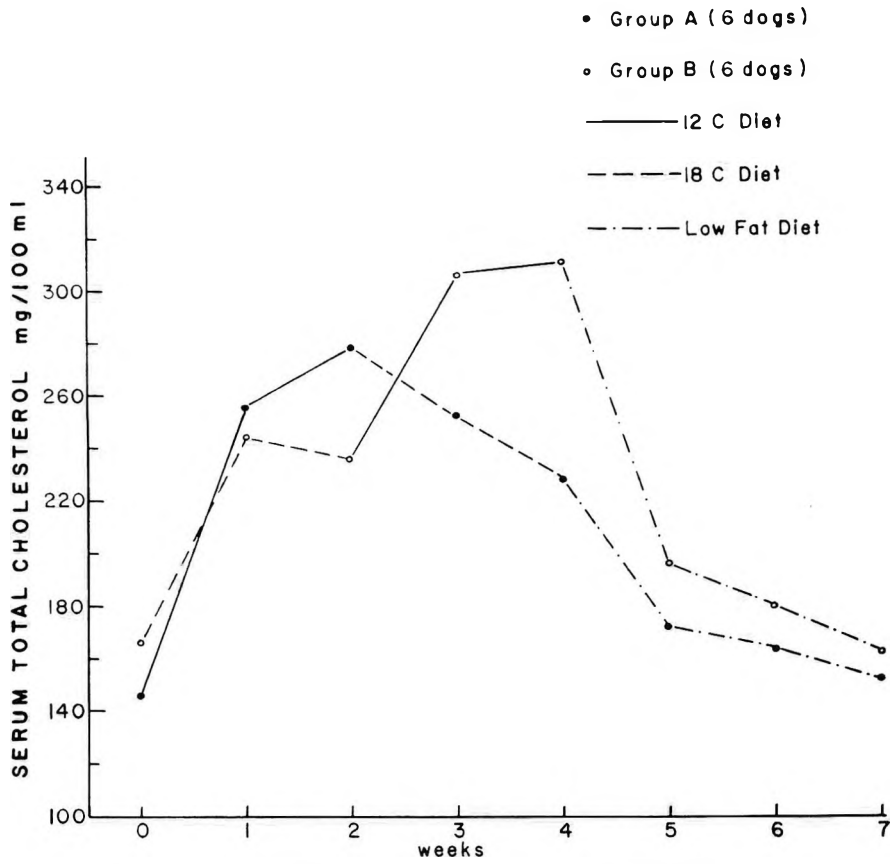

Fig. 2 The changes of serum total cholesterol in experiment 30. Values are means for the 6 dogs of each group.

but 12C produced higher cholesterol levels. This became clearly apparent when the diets were exchanged. The dogs changing from mixtures 12C to 18C showed decreases of serum cholesterol, with the dogs making the opposite change showing increases. Similar changes were observed in serum phospholipids and total fatty acids.

The analysis of the serum lipid changes is presented in table 5. The 12C diet caused significantly higher serum cholesterol, phospholipids and total fatty acids than the 18C diet. No significant differences in serum triglyceride level were noted between the two experimental diets. In contrast with the other lipid fractions, the triglycerides were higher with the low-fat diet than with the experimental diets. The mean of the individual differences between the triglyceride levels in control and on the experimental diets (average of the levels for diet 12C and 18C) was 26 mg per 100 ml (SE of the mean $\pm$ 7.37) which was significant ($P = 0.005$).

All the animals ate well in this experiment. The mean food intake for the 12C diet was 80 Cal. per kg per day and for the 18C diet, 83 Cal. per kg per day. The body weights were constant.

Stools were collected from 4 animals (two of each group) for the last three days of each experimental period. The mean fat content (as fatty acids) of the 4 stool samples collected when diet 12C was fed was 0.98 gm (SE of the mean $\pm$ 0.37) per day. The corresponding value for the 18C diet was 7.26 gm (SE of the mean $\pm$ 0.37) per day. The difference (6.28 gm per day, SE of the mean $\pm$ 0.60) was significant at

the level of $P < 0.005$. The content of digitonin precipitable, Liebermann-Burchard reacting material of the stools was equivalent to 114 mg of cholesterol per day (SE of the mean ± 21.7) for the 12C diet and to 234 mg per day (SE of the mean ± 50.3) for the 18C diet. The mean difference, 120 mg per day (SE of the mean ± 40.8), was not significant ($P = 0.06$).

The effects of the various fats used in these experiments on serum cholesterol concentration can be compared using the cholesterol values with the low-fat diet as a reference, because the same low-fat diet was used in all of them. Such a comparison is presented in table 6, which incorporates the results of the present experiments and also those of two other experiments (exp. 24 and 25) in which the same amount of coconut oil was administered (20% of the diet weight) for the same period of time (two weeks) and using the same low-fat diet. The values in the column headed "Cholesterol elevation" are means of the differences between the cholestrol values at the end of two weeks of feeding the experimental diet, and the mean cholesterol values with the low-fat diet, computed using the values of the two control (low-fat) periods.

Coconut oil and the 12C mixture produced the greatest elevations of serum cholesterol, followed by the 18C mixture, by MCT and by the MCT mixture, in that order.

The elevation of serum cholesterol produced by the administration of coconut oil to the dog was related to the intrinsic cholesterol level of the individual animal. The coefficient of correlation between the

TABLE 5

The serum lipids in experiment 30[1]

	Diets			Difference 12C *minus* 18C	
	Low-fat (control 1)	12C mixture	18C mixture		
Total cholesterol, mg/100 ml	156 ± 14.8	294 ± 14.9	233 ± 11.7	61 ± 9.2	< 0.0001
Phospholipids, mg/100 ml[2]	266 ± 22.6	509 ± 21.8	426 ± 20.1	83 ± 16.2	0.0002
Total fatty acids, mEq[3]	11.3 ± 0.65	19.3 ± 0.85	15.9 ± 0.56	3.4 ± 0.55	< 0.0001
Triglycerides mg/100 ml[4]	52 ± 5.8	28 ± 8.2	25 ± 4.4	3 ± 9.3	0.8

[1] Values are means and standard errors of the means for 12 dogs.
[2] Phospholipids = lipid phosphorus × 25.
[3] Total fatty acids determined by the method of Albrink ('59).
[4] Triglycerides, mg/100 ml = (285.7) [total fatty acids, mEq/100 ml − (0.0646 lipid P mg/100 ml) − (0.0019 total cholesterol, mg/100 ml)].

TABLE 6

Elevation of serum total cholesterol above the level for the low-fat diet,[1] *produced by various fat supplements given for two weeks as 20% of the diet weight (40% of the total calories)*[2]

Exp. no.	No. of dogs	Fat supplement	Cholesterol elevation		P
			Mean	SE mean	
			mg/100 ml		
24,25,27,28	31	coconut oil	146	± 12.0	< 0.0001
30	12	12C mixture	137	± 9.9	< 0.0001
30	12	18C mixture	76	± 7.9	< 0.0001
27	7	MCT	47	± 22.9	0.09
28	12	MCT mixture	21	± 8.4	0.025

[1] Low-fat diet values used were the means for each animal for the low-fat diet periods, before and immediately after the administration of the experimental fats.
[2] Values are means and standard errors of the means.

serum cholesterol increase after two weeks of coconut oil and the cholesterol level with the low-fat diet (mean of the two low fat control periods) was 0.528, (SE ± 0.155) for 31 dogs. This correlation coefficient was significant ($P = 0.002$).

DISCUSSION

The preceding data show that the glycerides of saturated fatty acids of 12- and 14-carbon atoms produce higher levels of serum cholesterol and phospholipids than the glycerides of saturated fatty acids of either 16- and 18-, or 8- and 10-carbon atoms. On comparison with the low-fat diet, the 12- and 14-carbon saturated fatty acids produced the greatest increase of cholesterol and phospholipids. The 8- and 10-carbon fatty acids produced the smallest increase with the acids of 16 and 18 carbons producing intermediate elevations. This result is in general qualitative agreement with the observations in man (Beveridge et al., '59; Hashim et al., '60; Grande et al., '61).

In other respects, however, the results obtained in the dog differ from the results obtained in the human species. In the first place, the absolute value of the cholesterol differences is greater in the dog than in man. The comparison between the 12C and the 18C mixtures showed in man a mean cholesterol difference of 8 mg per 100 ml, whereas in the present study, using fat mixtures similar in composition to those used in man, the difference observed was 61 mg per 100 ml (SE of the mean ± 9.2). It seems unlikely that the greater difference observed in the dog is due only to the higher proportion of fat in the diet, which was 40% of the total calories in the dog's experiment as compared with 30% in the human experiment (Grande et al., '61).

Secondly, the dog appears to differ from man in that the lowest cholesterol values are observed when the dog is fed the low-fat diet. The isocaloric replacement of carbohydrate by corn oil in the proportion of about 30% of the total calories produced in man a decrease of serum cholesterol of 23 mg per 100 ml (Anderson et al., '60); whereas in the dog the addition to the low-fat diet of sunflower oil in the proportion of 40% of the total calories produced serum cholesterol levels 29 mg higher than the low-fat diet (Grande et al., '60). This elevation of serum cholesterol, which had a probability of chance occurrence of $P = 0.035$, is of the same order of magnitude as the cholesterol elevation produced by the MCT mixture in experiment 28. Such observations indicate that MCT mixture and the linoleic acid-rich sunflower oil produce comparable serum cholesterol levels in the dog. In this respect the results in the dog are similar to those obtained in man by Hashim et al. ('60). These workers observed that MCT produces in man serum cholesterol levels about 10% above those observed with corn oil. The glycerides of saturated fatty acids of 8- and 10-carbon atoms therefore produce, both in man and in the dog, serum cholesterol levels of the same order as the polyunsaturated fatty acids. The triglycerides of saturated fatty acids of 8- and 10-carbon atoms apparently lack the known cholesterol-raising effect exerted by the triglycerides of the longer-chain saturated fatty acids.

The short-chain fatty acids differ from the long-chain fatty acids in their chemical properties, their form of transport in the body and their metabolic fate (Fritz, '61). Decanoic acid and the fatty acids with less than 10 carbons are transported from the intestine by the portal blood, and about 50% of the absorbed lauric acid is transported by the lymph. The proportion of absorbed fatty acid transported by the lymph increases with the length of the carbon chain of the fatty acid (Bloom et al., '51; Kiyasu et al., '52; Blomstrand, '55; Fernandes et al., '55). This difference in the route of transport from the intestine may be important in explaining the different effects of the two groups of saturated fatty acids, but will not explain the different effects of linoleic and long-chain saturated fatty acids both of which are transported by the lymph (Reiser and Bryson, '51). The fatty acids with shorter chain length than that of lauric acid are not incorporated into the fat depots (Longenecker, '39; Weitzel, '56; Fritz, '61) and their rate of oxidation in the body is faster than that of the longer-chain fatty acids, both in the fed and in the starved animal (Geyer et al., '51; Lossow and Chaikoff,

'55; Kirschner and Harris, '61). The fatty acids of 10 or less carbon atoms are rapidly oxidized by the animal body and do not burden the fat transport mechanisms as the longer-chain fatty acids do. This may be the reason for their lack of cholesterol-raising effect, but our studies do not provide a final answer to this question.

Recent reports indicate that the short-chain saturated fatty acids do not have the effect of increasing the requirement of essential fatty acids that the long-chain saturated fatty acids have (Kaunitz et al., '60). It may be speculated that this is the reason they do not increase the serum cholesterol concentration; but there is no proof that the cholesterol-raising effect of the long-chain saturated fatty acids is due to a relative deficiency of essential fatty acids (Portman et al., '61), and it has been shown that the cholesterol-depressing effect of the polyunsaturated fatty acids is independent of their activity as essential fatty acids (Anderson et al., '57; Keys et al., '57; Ahrens et al., '59).

In experiment 30 the 12C mixture produced significantly higher serum cholesterol and phospholipids than the 18C mixture. The slight mismatch in the composition of the two experimental fats (table 2) would tend to produce the opposite result. It is unlikely that the differences of cholesterol and phospholipid levels between the 12C and the 18C mixtures observed in this experiment can be explained by differences in the transport or the rate of oxidation of their saturated fatty acids. There is indication that the different effects of these two fat mixtures might be related to differences of intestinal absorption. The fat content of the feces was significantly higher with the 18C diet than with the 12C diet. This effect is probably due to the tristearin present in the totally hydrogenated corn oil incorporated into the 18C mixture. The fecal excretion of digitonin precipitable, Liebermann-Burchard reacting material was also higher for each dog fed on the 18C diet, but the mean difference was not statistically significant. The difference of sterol content between the two diets does not account for the different content of digitonin precipitable Liebermann - Burchard reacting material of the feces. The content of this material in the 12C mixture was equivalent to 30 mg of cholesterol per 100 gm and that of the 18C mixture to 48 mg. Using the food consumption data of the 4 animals the mean intake of digitonin precipitable, Liebermann-Burchard reacting material (including that of the low-fat basic diet) was observed to be equivalent to 32 mg of cholesterol per day for 12C and to 43 mg for 18C. The mean difference was only 11 mg per day as compared with a mean difference of 120 mg in fecal excretion.

The tendency of the 18C diet to produce higher fecal excretion of digitonin precipitable Liebermann-Burchard reacting material may be due to a decrease of the reabsorption of biliary cholesterol, or to an increase of the secretion of sterols by the bile or through the intestinal wall, or to a combination of these changes. Such effects, by increasing the rate of loss of cholesterol from the body, could be responsible for the decrease of serum cholesterol concentration.

SUMMARY

Fats of similar composition in terms of saturated, monoene, and polyene fatty acids, but differing in the chain length of the saturated fatty acids, were given alternately to groups of dogs at a level of 40% of the total calorie intake for periods of two weeks. Each experiment also included two control periods before and after the administration of the experimental fats, in which a low-fat diet containing 4% of fat calories was given.

The glycerides of saturated fatty acids of 12- and 14-carbon atoms produced significantly higher serum cholesterol and phospholipid values than either the glycerides of 16- and 18-carbon saturated fatty acids or of 8- and 10-saturated fatty acids.

When compared with the low-fat diet the experimental diets produced the following mean increases of serum cholesterol concentration (in mg per 100 ml ± SE of the mean): coconut oil, 146 ± 12.0; 12C mixture (60% of coconut oil, 40% of olive oil), 137 ± 9.9; 18C mixture (80% oleostock, 20% of totally hydrogenated corn oil), 76 ±7.9; MCT mixture (a synthetic triglyceride containing mainly saturated fatty acids of 8- and 10-carbon

atoms with 6% of olive oil and 2% of safflower oil), 21 ± 8.4. The serum phospholipid changes were parallel to those of cholesterol. The cholesterol levels produced by the glycerides of saturated fatty acids of 8- and 10-carbon atoms were similar to those produced in the dog by oils rich in polyunsaturated fatty acids (sunflower oil) given at the same proportion of the total calories.

ACKNOWLEDGMENTS

The author is indebted to Dr. V. C. Babayan for supplying the MCT used in these experiments, and to Drs. D. S. Amatuzio, J. T. Anderson, A. Keys and S. Wada for much valuable help during the studies. The technical help of Lois A. Laven, Sheila B. Quinn and Donna E. Swenson is gratefully acknowledged.

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Inhibitory Effect of Sulfaguanidine on the Biosynthesis of Thiamine in Rats and the Beneficial Role of Penicillin and Hydrolyzed Glucose Cycloacetoacetate

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Recent reports by many workers (Lih and Baumann, '51; Sauberlich, '52; Guggenheim et al., '53; Schendel and Johnson, '54; Jones and Baumann, '55; Johansson, '56) indicate that penicillin has a sparing effect upon the thiamine requirement of animals. On the other hand, Gant et al. ('43), Miller ('45) and Schonheyder ('46) observed that sulfonamides had an inhibitory effect on the biosynthesis of B vitamins. Sauberlich ('52) has reported that the addition of penicillin to the diet (0.01%) caused a marked stimulation in the growth of rats fed diets free of or low in B vitamins, whereas the addition of sulfamerazine even at a level of 0.5% of the diet had an inhibitory effect upon growth. Recent work of Balkrishnan et al. ('57) shows that the inhibitory effect of sulfaguanidine on the intestinal synthesis of thiamine could be overcome by feeding 13 gm of curds per rat daily together with sulfaguanidine in the thiamine-free basal diet. Scott and Griffith ('57) also observed the thiamine-sparing action of penicillin in weanling rats.

Recently, Barnes et al. ('60) studied extensively the thiamine-sparing action of penicillin and observed that coprophagy is essential for the availability of microbial-synthesized thiamine in penicillin-fed animals.

The investigations carried out in this laboratory show that hydrolyzed glucose cycloacetoacetate (GCA), which was found to contain 1:2 dienol glucose (Nath and Bhattathiry, '56) prevented not only experimental diabetes (Nath and Behki, '57, '58) and experimental hyperlipemia induced by feeding a high level of saturated fat or cholesterol (Nath and Saikia, '59a, b, c), but also stimulated biosynthesis of thiamine (Nath and Meghal, '60).

In this study an attempt was made to determine whether penicillin or hydrolyzed GCA would prevent the inhibitory effect of sulfaguanidine on the intestinal synthesis of thiamine in rats when either one was fed together with sulfaguanidine in the thiamine-inadequate basal diet.

EXPERIMENTAL

Forty-two male weanling albino rats (30 to 40 gm each) were fed a thiamine-inadequate diet for 5 days. Rats were caged individually and fed ad libitum. Coprophagy was not prevented during the experiment. Weights of animals were recorded weekly. The composition of the thiamine-inadequate basal diet is shown in table 1. After a 5-day depletion period, groups of 6 animals were subjected to the following regimens:

A Basal diet
B Basal diet + 5.6 mg thiamine·HCl per kg
C Basal diet + sulfaguanidine
D Basal diet + penicillin
E Basal diet + sulfaguanidine + penicillin
F Basal diet + hydrolyzed GCA
G Basal diet + sulfaguanidine + hydrolyzed GCA

Fifty milligrams of crystalline potassium penicillin G were added per kg to the diets of the groups receiving penicillin. Daily doses of sulfaguanidine and hydrolyzed GCA were 25 mg and 80 mg per rat, respectively.

Received for publication October 9, 1961.
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TABLE 1

Composition of basal diet

Major components	gm
Vitamin-free casein[1]	25
Dextrin	20
Groundnut oil	15
Sucrose	33
Salts[2]	4
Choline chloride	0.3
B vitamins in sucrose	2
Fat-soluble vitamins in corn oil	1
Total	100.3

B vitamins in 2.0 gm of sucrose	mg
Thiamine·HCl	0.04
Riboflavin	0.80
Pyridoxine·HCl	0.40
Ca pantothenate	4.00
Niacin	4.00
Inositol	20.00
Biotin	0.02
Folic acid	0.20
Vitamin B_{12}	0.03
Menadione	1.00

Fat soluble vitamins in 1.0 gm of corn oil	mg
Vitamin A acetate	0.31
Vitamin D	0.0045
α-Tocopheryl acetate	5.00

[1] Commercial casein extracted with acid alcohol for 4 hours.
[2] Hubbel et al. ('37).

The animals were fed the respective diets for 4 weeks. Urine and feces were collected the last three days of each week. Urinary thiamine content was determined according to the method of Mawson and Thompson ('48), and that of fecal thiamine by the method adopted by the Association of Vitamin Chemists ('51).

During the 4 weeks of experiment, the weekly fecal coliform count was taken of three rats from each group. The feces of each rat were collected aseptically on the last three days of the week in weighed sterile test tubes and a uniform suspension of about 0.1 gm of feces with 10 ml of saline (0.9% NaCl solution) was made under aseptic conditions. Further dilutions were made with the saline, and the coliform colonies in the feces were counted by the plate method using Mackonkey's agar medium (Balakrishnan and Rajagopalan, '52).

At the end of fourth week all animals were killed by a sharp blow on the head and their ceca and livers excised. The contents of each cecum were expelled into small glass-stoppered bottles and frozen immediately. Each liver was weighed and frozen and the thiamine content of each sample was determined by the method of Kratzing and Slater ('50). The cecal contents were weighed and digested in a flask with a sufficient amount of 0.1 N HCl; samples were then prepared in the recommended manner and total thiamine was estimated by the method of Hennessey and Cerecedo ('39).

GCA was prepared by condensing glucose with ethylacetoacetate as described by West ('27) and modified by Nath et al. ('52). It was hydrolyzed as follows.

In a 50-ml conical flask 2 gm of crystalline GCA were hydrolyzed with 2 N HCl over a boiling water bath for 20 minutes. The flask was cooled over ice and the contents were neutralized to about pH 7. The hydrolysate was extracted three times with ether; the aqueous fraction was separated and adjusted carefully to pH 7.2 with a few drops of 2 N NaOH, using Merck special indicator paper (pH range 6.6 to 8.0), then diluted with water so that it contained the equivalent of 80 mg of GCA per ml.

RESULTS

The daily urinary and fecal excretion of thiamine increased in the groups receiving penicillin and hydrolyzed GCA, either in combination with sulfaguanidine or alone (table 2). Sulfaguanidine, when administered at a dose of 25 mg per rat daily, decreased the urinary and fecal excretion of thiamine during the experiment; rats of this group showed symptoms of thiamine deficiency such as polyneuritis and loss of appetite. The increase in urinary and fecal excretion of thiamine gives an indication of thiamine synthesis. In group A (controls) although urinary and fecal excretion of thiamine decreased, the decrease was not as low as that in group C (sulfaguanidine-fed), thereby indicating that the intestinal synthesis was inhibited to a great extent in group C. However, it was found that this inhibitory effect of sulfaguanidine could be overcome by feeding either penicillin or hydrolyzed GCA in the basal diet.

TABLE 2

Effect of sulfaguanidine, penicillin and hydrolyzed glucose cycloacetoacetate (GCA) on the urinary and fecal excretion of thiamine

Group	Diet	Urinary excretion, in weeks				Fecal excretion, in weeks			
		1	2	3	4	1	2	3	4
		μg per 24 hours per rat				*μg per 24 hours per rat*			
A	Basal diet (control)	0.82 ± 0.02[1]	0.80 ± 0.03	0.75 ± 0.02	0.73 ± 0.03	1.21 ± 0.02	1.10 ± 0.06	1.00 ± 0.05	0.99 ± 0.06
B	Basal diet + 5.6 mg thiamine HCl/kg	1.22 ± 0.06	1.38 ± 0.05	1.42 ± 0.06	1.43 ± 0.02	2.31 ± 0.05	2.35 ± 0.03	2.57 ± 0.04	2.56 ± 0.06
C	Sulfaguanidine in basal diet	0.80 ± 0.03	0.62 ± 0.02	0.52 ± 0.02	0.50 ± 0.01	1.11 ± 0.03	0.98 ± 0.02	0.82 ± 0.03	0.76 ± 0.04
D	Penicillin in basal diet	1.18 ± 0.02	1.28 ± 0.03	1.30 ± 0.02	1.32 ± 0.05	2.30 ± 0.04	2.31 ± 0.07	2.45 ± 0.03	2.48 ± 0.04
E	Sulfaguanidine + penicillin in basal diet	0.93 ± 0.05	0.98 ± 0.02	1.00 ± 0.01	1.08 ± 0.02	2.00 ± 0.02	2.15 ± 0.03	2.05 ± 0.05	2.06 ± 0.08
F	Hydrolyzed GCA in basal diet	1.21 ± 0.02	1.20 ± 0.03	1.28 ± 0.05	1.26 ± 0.05	2.12 ± 0.01	2.18 ± 0.02	2.30 ± 0.06	2.31 ± 0.03
G	Sulfaguanidine + hydrolyzed GCA in basal diet	0.85 ± 0.02	0.97 ± 0.03	1.15 ± 0.02	1.16 ± 0.03	1.82 ± 0.05	1.91 ± 0.06	2.00 ± 0.02	2.02 ± 0.06

[1] Mean ± standard deviation.

TABLE 3

Effect of different treatments on liver and cecal thiamine content of 6 rats

Group	Treatment	Total liver[1] thiamine	Liver thiamine[1]	Total cecal[1] thiamine
		μg	*μg/gm*	*μg*
A	Basal diet (control)	2.17 ± 0.02[2]	1.22 ± 0.04	2.16 ± 0.08
B	Thiamine-supplemented basal diet	14.40 ± 0.07	2.80 ± 0.07[3]	2.87 ± 0.08
C	Sulfaguanidine in basal diet	1.32 ± 0.08	0.33 ± 0.02	1.31 ± 0.01
D	Penicillin in basal diet	10.5 ± 0.32	2.51 ± 0.04[3]	2.58 ± 0.14
E	Sulfaguanidine + penicillin in basal diet	4.22 ± 0.18	1.44 ± 0.04[3]	2.12 ± 0.07
F	Hydrolyzed GCA in basal diet	6.03 ± 0.60	1.51 ± 0.04[3]	2.58 ± 0.14
G	Sulfaguanidine + hydrolyzed GCA in basal diet	4.20 ± 0.59	1.46 ± 0.07[4]	2.20 ± 0.24

[1] On wet-weight basis.
[2] Standard error of the mean.
[3] Significant difference from group A at $P < 0.01$.
[4] Significant difference from group A at $P < 0.02$.

In table 3 are presented data showing the hepatic and cecal thiamine content. The thiamine content of liver was higher in groups receiving penicillin, hydrolyzed GCA and sufficient thiamine and these values are statistically significant ($P < 0.01$) when compared with controls. The hepatic level of thiamine is significantly reduced in group C ($P < 0.01$); but feeding of either penicillin or hydrolyzed GCA along with sulfaguanidine in the basal diet maintained the hepatic storage of thiamine to a significant level ($P < 0.01$). The cecal thiamine content increased in all groups except in group C where it was decreased.

The weekly weights of animals in the different groups are shown in figure 1. The thiamine-sparing action of penicillin or hydrolyzed GCA is evidenced by a constant increase in the body weights of rats in these groups, whereas the sulfaguanidine-fed group shows a decrease in weight throughout the experiment. However, it was found that this inhibitory effect of sulfaguanidine could be overcome by penicillin or hydrolyzed GCA because these groups did not lose body weight.

In figure 2 are presented the weekly fecal coliform counts. Sulfaguanidine alone decreased the coliform count, which was lowest in the fourth week.

Hydrolyzed GCA increased the fecal coliform count throughout the experiment and

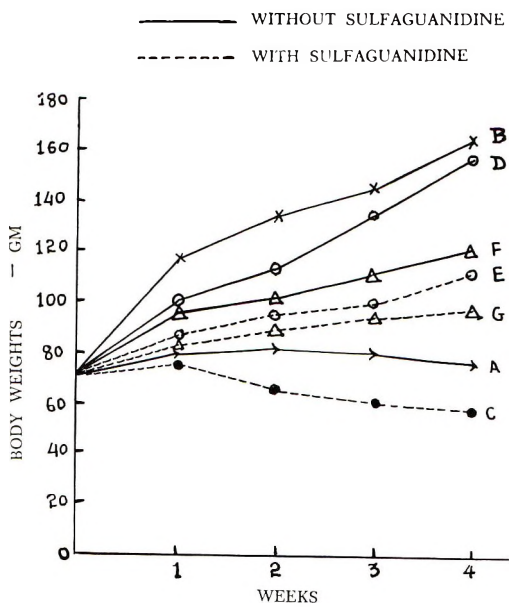

Fig. 1 Average growth rate of rats during the experiment.

Group A, fed basal diet (control)

Group B, fed thiamine-supplemented basal diet

Group C, fed sulfaguanidine in basal diet

Group D, fed penicillin in basal diet

Group E, fed sulfaguanidine + penicillin in basal diet

Group F, fed hydrolyzed glucose cycloacetoacetate (GCA)

Group G, fed sulfaguanidine + hydrolyzed GCA

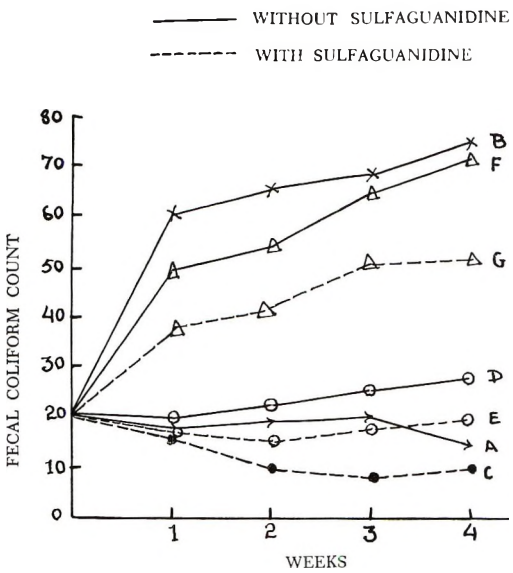

Fig. 2 Total fecal coliform count during the experiment; average values expressed as 10^{-4} per gram of wet feces.

Group A, fed basal diet (control)

Group B, fed thiamine-supplemented basal diet

Group C, fed sulfaguanidine in basal diet

Group D, fed penicillin in basal diet

Group E, fed sulfaguanidine + penicillin in basal diet

Group F, fed hydrolyzed glucose cycloacetoacetate (GCA)

Group G, fed sulfaguanidine + hydrolyzed GCA

prevented the inhibitory action of sulfaguanidine on the coliform count. It appears that penicillin had no appreciable effect on coliform count.

DISCUSSION

Oral administration of penicillin has been observed to spare thiamine in rats (Lih and Baumann, '51; Sauberlich, '52; Guggenheim et al., '53; Schendel and Johnson, '54) and various suggestions have been made as to the mechanism of the thiamine-sparing action of penicillin. For example penicillin may spare thiamine by (1) increasing its absorption from the diet, (2) preventing its bacterial destruction or utilization in the tract, or (3) increasing its intestinal synthesis. But recent reports of Mameesh et al. ('59) and Barnes et al. ('60) show that penicillin is effective in sparing thiamine only when coprophagy is allowed; they have further shown that penicillin increases fecal excretion of thiamine which is available to animals after coprophagy. Our results also confirm the observations of these workers. Penicillin increased the urinary and fecal excretion of thiamine, and the storage of thiamine in the liver. This indicates that penicillin stimulated intestinal synthesis of thiamine that was available only after coprophagy.

This action of penicillin may be due to the increase in intestinal flora and decrease in the thiamine utilizing or destroying bacteria in the intestine, because penicillin does not spare thiamine when injected intraperitoneally (Guggenheim, '53; Johanssen, '57).

The inhibitory effect of sulfaguanidine on the biosynthesis of thiamine was observed in this study also. Sulfaguanidine suppresses the growth of coliform organisms which are known to be important in the synthesis of thiamine. The inhibitory effect of sulfaguanidine in thiamine biosynthesis has been reported by Balakrishnan et al. ('57), who showed further that this effect of sulfaguanidine could be overcome by feeding curds to rats. In the present study the effect of penicillin in overcoming the inhibiting effect of sulfaguanidine has also been demonstrated.

Hydrolyzed GCA which has already been reported to enhance the biosynthesis of thiamine (Nath and Meghal, '60) and prevent the thiamine deficiency caused by feeding glucose or sucrose at a 10% level in carbohydrate - free thiamine - deficient diets (Nath and Meghal, '61), was found to spare thiamine in the presence of sulfaguanidine. Rats in this group showed a higher cecal coliform count with an increase in the urinary and fecal excretion of thiamine, thus enhancing thiamine synthesis. Thiamine thus synthesized by intestinal flora is excreted in the feces and is available to animals after coprophagy (Barnes et al., '60).

SUMMARY

When rats were fed sulfaguanidine together with a thiamine-low basal diet, urinary and fecal thiamine excretion decreased, and the animals showed symp-

toms of thiamine deficiency; thus an inhibitory effect of sulfaguanidine on the intestinal synthesis of thiamine was indicated. This inhibitory effect could be overcome by feeding either penicillin or hydrolyzed glucose cycloacetoacetate. Rats thus fed showed an increase in the urinary and fecal excretion of thiamine, with a higher thiamine content in the liver. Inclusion of either penicillin or hydrolyzed glucose cycloacetoacetate promoted better growth with the use of thiamine-low diets. This observation indicates that both compounds enhanced the thiamine synthesis by the intestinal flora and caused an increase in fecal thiamine, which was ultimately available to the rats after coprophagy.

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Effect of Purified Diets on the Fatty Acid Composition of Sheep Tallow[1,2]

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During an investigation (Matrone et al., '57, '59) of purified diets for ruminants, it was observed that a sample of rendered fat obtained from one of the animals on experiment remained liquid at room temperature. Analysis of the fatty acid composition of this sample revealed that the level of stearic acid was considerably lower than that reported for sheep tallow (Hilditch and Pedelty, '41; Brooker and Shorland, '50). Since the purified diet contained 4% of fat, the low level of stearate was even more unexpected inasmuch as the usual fate of dietary fatty acids in ruminants is hydrogenation resulting in increased stearic acid levels in the depot fat (Willey et al., '52; Garton, '60; Tove, '60).

Following this initial observation the investigation reported herein was undertaken to: (a) further characterize the depot fat of sheep fed purified diets and (b) determine, if possible, the cause of the unusual fatty acid composition of depot fats associated with these diets.

EXPERIMENTAL

For the characterization phase, samples of inguinal subcutaneous adipose tissue were obtained by biopsy from 30 sheep fed various purified diets (Matrone et al., '57, '59), all of which basically involved combinations of the following ingredients: casein, glucose, starch, hydrogenated vegetable fat,[3] salts of volatile fatty acids, glycerol, triacetin, vitamins,[4] and minerals plus sodium and potassium bicarbonates. Control samples were obtained from 4 normal sheep on pasture.

The objective of the second phase of the investigation was to determine the effect of the dietary fat on the fatty acid composition of the depot fat, particularly since the hydrogenated vegetable fat was common to all purified diets. An additional reason for checking out this dietary fat was that it was known to contain large quantities of transisomers. This experiment was conducted with 8 lambs with an average initial weight of approximately 50 pounds. It involved two replications of a 2×2 factorial design in which the variables were (1) 4% of lard compared with 4% of hydrogenated vegetable fat, and (2) a casein-glucose-starch purified diet supplemented with sodium and potassium bicarbonates, similar to diet 1 (Na plus K) (Matrone et al., '59), compared with a diet containing 50% of alfalfa hay and 46% of grain (made up of equal parts of corn and oats). Three samples of inguinal subcutaneous adipose tissue (0.2 to 1.0 gm) were taken by biopsy from each animal: the first was taken 6 weeks after initiation of the experiment and the other two at three-week intervals thereafter. At the end of the experiment a 30-gm sample of mesenteric adipose tissue was obtained from each animal by biopsy for analyses of transisomers. At the termination of the experiment samples of rumen fluid from each animal also were taken at predetermined intervals for fatty acid analyses.

The samples of inguinal subcutaneous adipose tissue were homogenized with 20 ml of a mixture of alcohol and ether

Received for publication November 2, 1961.

[1] Contribution from the Animal Industry Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper no. 1384 of the Journal Series.

[2] Supported in part by funds from the U. S. Public Health Service, Grant A-2483, and a grant from the Herman Frasch Foundation.

[3] Primex BC, hydrogenated vegetable fat, kindly donated by The Procter and Gamble Company, Cincinnati.

[4] The authors wish to thank Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the biotin; the R. P. Scherer Corporation, Detroit, for vitamins A and D; and Merck and Company, Inc., Rahway, New Jersey, for all other vitamins.

(3:1). The homogenates were brought to a boil, filtered through a sintered glass funnel and the solvents removed on a steam bath. The methyl esters of the fatty acids were obtained by transesterification in a medium consisting of 20 ml of methanol containing 5% of hydrogen chloride, 1 ml of 2,2-dimethoxypropane and sufficient benzene (2 to 5 ml) to form a homogeneous solution. The solution was allowed to remain overnight at room temperature, and the methyl esters of the fatty acids were extracted with hexane and thoroughly washed with water.

Samples of rumen fluid were saponified with KOH, the fatty acids isolated and converted to the methyl esters by means of the same procedure used for transmethylation.

The fatty acid composition was determined by gas chromatography on a 4-foot column of succinate-ethylene glycol polyester on Celite (Tove and Smith, '60).

Analysis of transisomers was carried out by measuring the infrared absorption, at 10.34 μ, of the free fatty acids dissolved in carbon disulfide. Elaidic acid[5] was used as a standard.

RESULTS

The striking contrast in physical consistency between the sheep fat on which the original observation was made and tallow from a normal animal is shown in figure 1.

The fatty acid analysis of the biopsy samples from the sheep fed the various purified diets revealed no significant differences in fatty acid composition that were associated with a particular diet. However, the fatty acid composition of all of the animals fed the purified diet was different from that of the normal animals (table 1). The percentages of stearic acid and palmitic acid were lower in the sheep fed the purified diets, and these were counterbalanced by corresponding increases in the levels of oleate and palmitoleate. The fatty acid composition of the normal animals was in agreement with that reported by other investigators (Hilditch and Pedelty, '41; Brooker and Shorland, '50).

The average gain of lambs fed the purified diets in the factorial experiment was

[5] Mann Research Laboratories, Inc., New York.

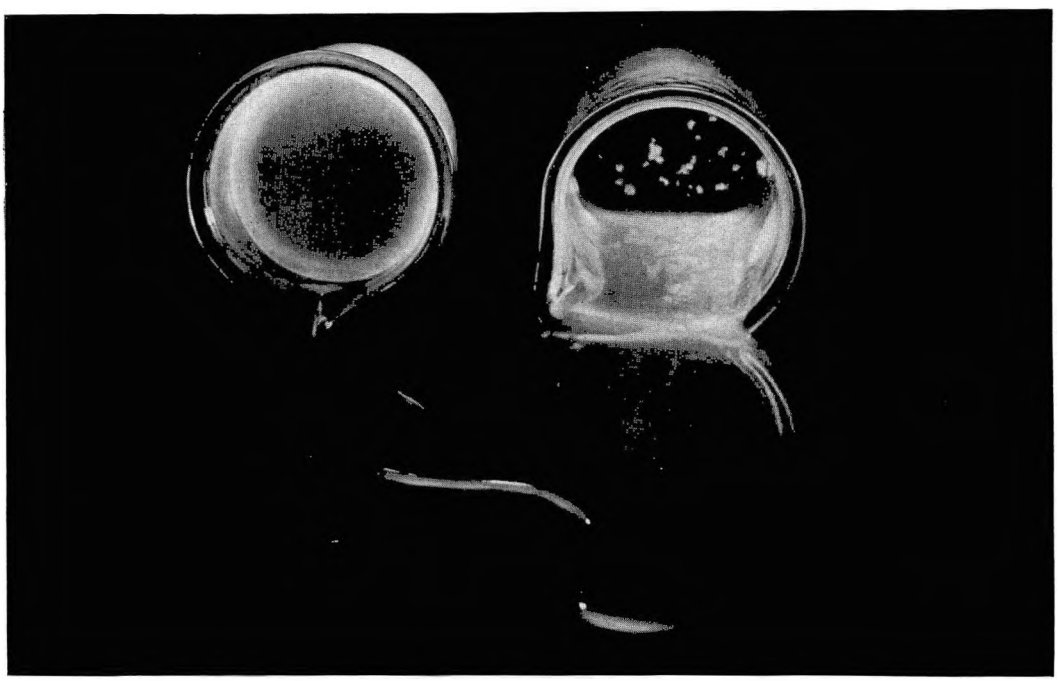

Fig. 1 Tallow from normal and purified-diet-fed sheep. The liquid tallow is the sample on which the effect of a purified diet on sheep tallow was first observed.

0.36 pounds per day; for those fed the hay-grain diet, 0.31 pounds per day.

The fatty acid analyses of the samples of inguinal subcutaneous adipose tissues of these animals are shown in table 1. Averages of the three tissue samples obtained from each animal are presented since there was little change in the fatty acid composition among time intervals. Two dietary effects on the fatty acid composition of the depot fat were observed. The more apparent of these was that the reduced percentage of stearate was associated with the purified diets, and that the type of fat fed had no effect on the level of this acid even though the percentage of stearic acid in the dietary fats varied widely (table 2). But the level of the 18-carbon monoenoic acids in the tallows from the sheep fed both purified and hay diets with hydrogenated vegetable oil was higher than the levels in the depot fat of the corresponding lard-fed animals. The higher levels of the 18-carbon monoenoic acids were counterbalanced by lower levels of palmitate. Moreover, the percentages of the 18-carbon monoenoic acids and palmitic acid in the sheep tallows reflected the levels of these acids in the dietary fats (table 2).

The average fatty acid composition of the samples of mesenteric adipose tissue is shown in table 3. Unlike the inguinal samples (table 1), the level of total 18-carbon monoenoic acids in the mesenteric fat of the animals fed the hay diets was somewhat lower than the levels in the corresponding sheep fed the purified diet. The levels of transisomers in the depot fats are clearly indicative of the deposition of these acids from the dietary fat. Moreover, when the total 18-carbon monoenoic acids were corrected for the transisomers, the differences in oleic acid content associated with the 4 diets became accentuated. With the exception of the 18-carbon monoenoic acids, essentially the same pattern of fatty acid deposition was observed with the mesenteric fat (table 3) as with the inguinal subcutaneous fat (table 1); however, the dietary differences were far more striking with the mesenteric tissue. This was particularly noticeable with stearic acid where the level in the mesenteric fat of the hay-fed animals was

TABLE 1

Fatty acid composition of inguinal subcutaneous adipose tissue of normal sheep and sheep fed purified diets

Diet	No. of sheep	Fatty acid[1]					
		14:0	14:1	16:0	16:1	18:0	18:1	18:2
		%	%	%	%	%	%	%
Normal	4	3.5 ± 0.39[2]	1.1 ± 0.44	28.9 ± 1.10	4.7 ± 0.61	18.7 ± 0.67	42.8 ± 1.92	2.0 ± 0.25
All purified	30	3.7 ± 0.11	1.3 ± 0.13	25.3 ± 0.41	6.9 ± 0.32	13.7 ± 0.43	47.6 ± 0.75	2.4 ± 0.31
Purified-vegetable	2[3]	3.6 ± 0.20	0.7 ± 0.08	24.4 ± 0.91	4.9 ± 0.34	15.7 ± 0.62	49.9 ± 1.03	0.7 ± 0.10
Purified-lard	2[3]	3.8 ± 0.21	0.8 ± 0.04	28.4 ± 0.48	4.5 ± 0.34	16.0 ± 0.60	45.8 ± 0.41	0.8 ± 0.09
Hay-vegetable	2[3]	2.6 ± 0.06	0.5 ± 0.05	23.4 ± 1.13	2.4 ± 0.39	21.7 ± 0.10	47.7 ± 1.51	1.3 ± 0.36
Hay-lard	2[3]	2.3 ± 0.13	0.7 ± 0.09	26.8 ± 0.61	3.9 ± 0.40	23.5 ± 0.42	41.3 ± 0.83	1.5 ± 0.11

[1] The first number refers to the number of carbon atoms; the second, to the number of double bonds.
[2] Standard error of the mean.
[3] Average of three samples taken by biopsy at three-week intervals.

TABLE 2

Fatty acid composition of the dietary fats

Diet	Fatty acid[1]									
	14:0	14:1	16:0	16:1	18:0	18:1			18:2	18:3
						Total	*Cis*	*Trans*[2]		
	%	%	%	%	%	%	%	%	%	%
Purified-vegetable	1.6	0.1	17.5	0.6	8.5	68.8	32.2	36.6	2.7	—
Purified-lard	2.0	0.1	27.7	3.2	16.6	42.5	41.2	1.3	6.6	1.2
Hay-vegetable	0.4	0.6	13.8	trace	4.4	54.4	—	—	24.5	1.7
Hay-lard	1.5	0.3	26.2	1.5	9.2	39.2	—	—	20.1	1.9

[1] The first number refers to the number of carbon atoms; the second, to the number of double bonds.
[2] All of the transisomers were assumed to be 18-carbon monoenoic acids.

TABLE 3

Fatty acid composition of mesenteric adipose tissue of sheep

Diet	Fatty acid[1]							
	14:0	16:0	16:1	18:0	18:1			18:2
					Total	*Cis*	*Trans*[2]	
	%	%	%	%	%	%	%	%
Purified-vegetable	2.1	21.6	5.0	18.7	51.0	30.7	20.3	0.8
Purified-lard	3.3	26.2	4.6	22.1	42.1	38.1	4.0	1.2
Hay-vegetable	1.9	19.6	1.9	40.2	33.7	17.1	16.6	1.8
Hay-lard	1.6	24.2	2.6	39.0	30.6	22.6	8.0	1.3

[1] The first number refers to the number of carbon atoms; the second, to the number of double bonds.
[2] All of the transisomers were assumed to be 18-carbon monoenoic acids.

double that of the sheep that received the purified diets.

Reduced hydrogenation of unsaturated fatty acids by the rumen microflora of the sheep fed the purified diets was also evident in the fatty acid composition of the rumen contents. In general the same patterns of fatty acid distribution associated with the 4 diets that were observed for the sheep tallow were noted for the rumen lipids (fig. 2). The level of stearic acid in the rumen fat of the sheep fed the hay diets was appreciably higher than the level in the rumen lipids of the animals fed the purified diet. The level of 18-carbon monoenoic acids in the rumen fat of the animals ingesting a purified diet also exceeded that of the corresponding hay diet. In addition, the level of 18-carbon monoenoic acids was also observed to be associated with the dietary fat, probably reflecting the content of transisomers in the dietary fats. Perhaps the most striking feature of these data, however, was the low percentage of linoleic acid in the rumen fat (fig. 2). This was particularly unexpected for the samples taken at one and three hours when the animals were ingesting fat containing as much as 20% of linoleic acid. The eightfold difference between the dietary and rumen levels of linoleic acid is indicative of the rapidity of the hydrogenation by the rumen microflora of the animals fed the hay-grain diets.

DISCUSSION

Although dietary unsaturated fatty acids are readily deposited in the depot fat by simple-stomach animals, the presence of unsaturated fatty acids in the diet of ruminants is accompanied by an increased deposition of saturated fatty acids by these animals (Willey et al., '52; Tove, '60). This species difference results because the dietary unsaturated acids are hydrogenated by the microorganisms that inhabit the rumen[6] (Shorland et al., '57; Hoflund et al., '56). If fat is administered parenterally, bypassing the rumen, the unsaturated acids of the injected fat are found in the animal fat (Tove, '60). Similarly, if rumen hydrogenation was reduced, a decreased level of stearate and

[6] Reiser, R. 1951 Hydrogenation of polyunsaturated fatty acids by the ruminant. Federation Proc., *10*: 236 (abstract).

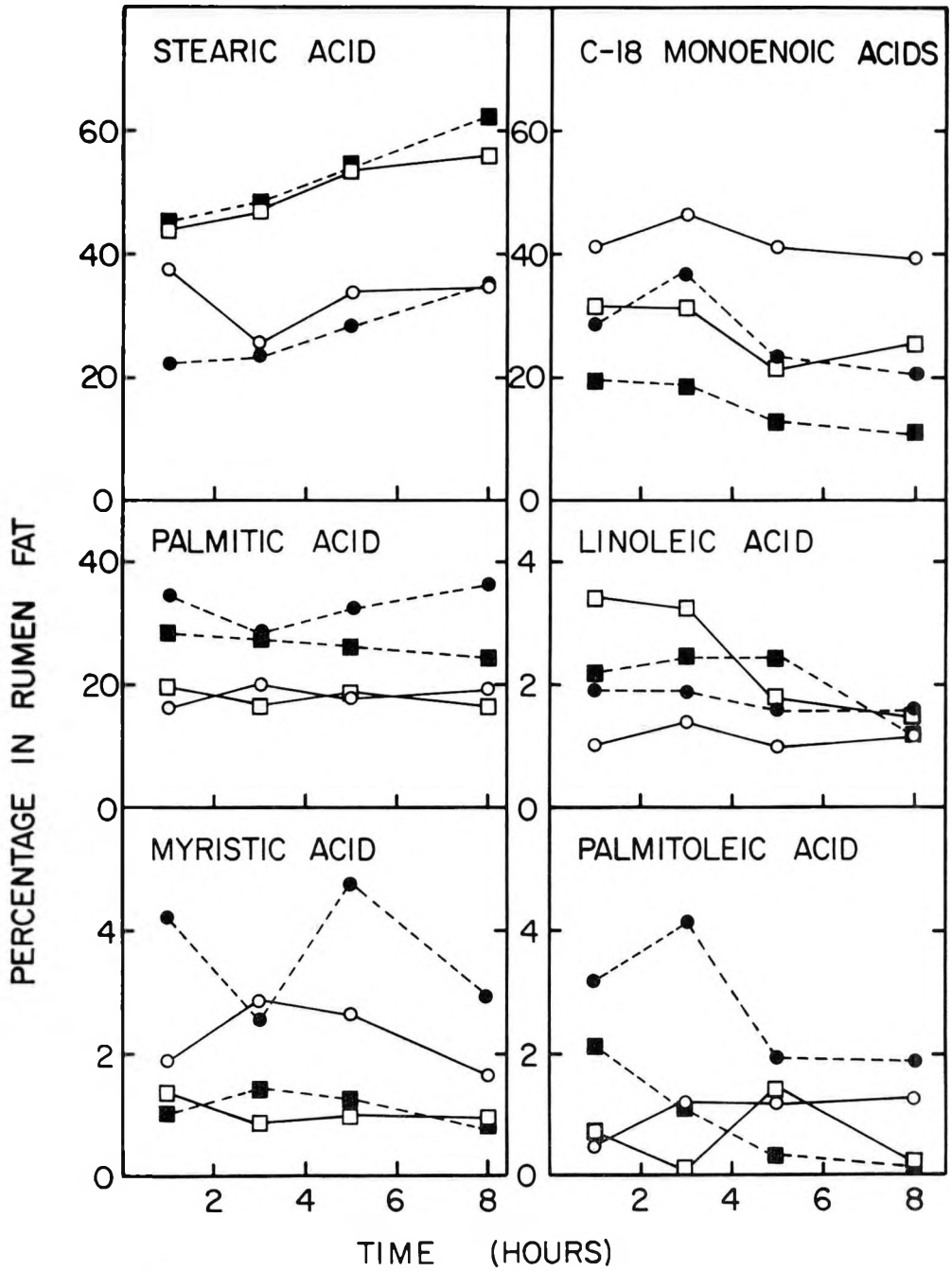

Fig. 2 Fatty acid composition of rumen lipids of sheep fed purified or hay diets: ○, purified diet with hydrogenated vegetable oil; ●, purified diet with lard; □, hay diet with vegetable oil; ■, hay diet with lard. Diets were fed ad libitum for three hours, then removed.

an increased level of unsaturated fatty acids in the depot fat might be expected. The observations reported herein are consistent with this thesis. Sheep that were fed the purified diets deposited more oleate and less stearate than hay-fed animals or normally grazing animals. Moreover, similar differences were noted for the percentages of these acids in the rumen lipids of sheep fed the purified diets as contrasted with the rumen lipids from hay-fed animals. Conversely, the level of stearate in the dietary fat bore no relation to levels of this acid in either the depot fat or rumen lipid, nor was the level of oleate in the dietary fat correlated with its content in depot fat.

The high content of soluble carbohydrate in the purified diets appears to be the most likely feature common to all of these diets that could account for the observed reduced hydrogenation of the unsaturated fatty acids. Its mode of action is unknown, however, although it is probably indirect. The rumen flora of the animals fed the purified diet has been shown to be morphologically different from that of normal animals.[7] Examination of the rumen fluid of the animals studied in the characterization phase revealed a specialized flora of large, gram positive streptococci and diplococci; fermentation gas analyses showed CO_2, CH_4 and no H_2 for all animals fed roughage-free purified diets. Thus it is possible that the hydrogenation capacity of these organisms is reduced because of a lowered activity of a fatty acid saturase, reduced concentration of a hydrogen donor compound or production of an inhibitor of the saturase. Which of these possibilities is involved, if any, remains for future experimentation. Since the linoleic acid content of the sheep tallows from the lard-fed animals was the same as that from those fed the vegetable fat, it is clear that hydrogenation of linoleic acid was carried out by the flora associated with the purified diets. Recently, Shaw et al. ('60) reported that the iodine number of tallow from steers fed finely ground pelleted hay and steamed corn was less than that from steers fed chopped hay and ground corn. They also noted an altered rumen fermentation as evidenced by a change in the proportions of acetate and propionate produced. Since steam heating the corn would increase its soluble carbohydrate, these observations are not inconsistent with those reported herein.

Transisomers of unsaturated fatty acids occur in relatively large amounts in the depot fat of ruminants (Hartman et al., '54) and the source of these acids has been shown to be the rumen contents with the development of the transacids accompanying the partial hydrogenation of the unsaturated fatty acids (Shorland et al., '57). The hydrogenated vegetable fat used in the study described in this report contained appreciable quantities of transisomers that were deposited in the tallows of sheep fed this fat. Since the same levels of transacids were observed in the depot fat of sheep fed either the purified-vegetable fat diet or hay-vegetable fat diet, it would seem apparent that transisomers of unsaturated fatty acids are resistant to hydrogenation.

SUMMARY

The tallow from sheep fed purified diets contained less stearate and more oleate than tallow from either sheep fed hay-grain diets or grazing animals. In some instances, the altered fatty acid distribution was sufficient to result in a tallow that was a liquid at room temperature. It is probable that less hydrogenation of unsaturated fatty acids was carried out by the rumen microorganisms of the sheep fed the purified diet, because the stearate level of the lipid of the rumen contents was appreciably less than that of the hay-fed animals. A high content of transacids in the dietary fat resulted in high levels of transacids in the sheep tallow irrespective of the remainder of the diet, indicating the resistance of the transisomers to hydrogenation by the rumen flora.

ACKNOWLEDGMENTS

The authors wish to thank E. L. Thomason, Jr., and Mrs. Anne Cannon for their technical assistance.

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Effect of Restricting the Hours of Feeding Upon Dental Decay in the Rat[1]

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Several investigators have reported a reduction in dental decay when the diets of experimental animals (rats and hamsters) were restricted to a definite percentage of the diet consumed by the controls. Shaw ('50), using white rats, fed three groups of 8 rats each (1) a cariogenic diet ad libitum, (2) 60% of the diet eaten by the control in group 1, and (3) 40% of the weight of the diet eaten by a suitable control in group 1. The rats restricted to 40% of the diet eaten by the controls showed a significantly low average incidence of dental caries, whereas those consuming 60% had less caries but not a statistically significant lower dental caries rate than their control littermates. He states these results are comparable to those he found for cotton rats.

Bixler and Muhler ('58), using a coarse corn diet, showed a marked reduction in dental caries when rats were restricted to 60% and to 40% of the daily food consumption of the control rats fed ad libitum. Dalderup and Jansen ('56) suggest that the reduction in caries with restricted feeding is a result of the food remaining in the mouth for a shorter time rather than a smaller intake of food.

Bibby ('55), after reviewing the work of others and presenting his studies with humans, concluded that "the frequency of eating carbohydrate or sugar-containing foods is a most important factor in determining the caries activity of foods."

The outstanding work of Lundqvist ('52) on oral sugar clearance using the human subjects of the Vipeholm study in Sweden (Gustafsson et al., '54) is probably the most extensive research in this field. Among other conclusions he states, "a high sugar concentration in the saliva, in conjunction with a prolonged clearance time, entails increased caries activity." He points out the value of rinsing the mouth with water after meals or after taking any foods between meals.

Of great interest is the report of Weiss and Trihart ('60) who studied the between-meal eating habits of 783 preschool children whose mean age was approximately 5¾ years. They found a "direct and consistent relationship" between the number of defective teeth and the frequency of eating "sweet" or "sticky" items, or both, between meals. Children who reported no such items between meals had 3.3 def[2] teeth per child, whereas those who reported 4 or more had 9.8 def teeth per child. Prior to the appearance of that report, the feeding experiments with rats here reported had been completed.

The paper which follows reports the results of restricting the consumption of a cariogenic diet for rats on a time basis, rather than on a percentage of the food eaten by the control rats, and noting the effect upon dental decay.

EXPERIMENTAL

The mothers of all rats received semi-natural diet 15 consisting of the following in grams: skim milk powder, 350; whole wheat flour, "fine grind," 650; cornmeal, 150; white flour (enriched), 150; brown rice flour, 150; soybean flour, 100; yeast, 30; cottonseed oil,[3] 46; cod liver oil, 4; salt mixture, 10; and iodized salt, 10; all ground to pass a 28-mesh sieve (Miller and Schlack, '58). For all dental caries experiments here reported, all ingredients

Received for publication October 6, 1961.

[1] Published with the approval of the Director of the Hawaii Agricultural Experiment Station as Technical Paper no. 547.

[2] def indicates decayed, indicated for extraction, and filled teeth.

[3] Wesson Oil, Wesson Oil Company, New Orleans, Louisiana.

for diet 15 were ground to pass through a 48-mesh sieve. Diet 18 was the same as diet 15 except that 17% of the whole wheat flour was replaced by powdered sucrose containing 3% of cornstarch, and again all ingredients were fine enough to pass through a 48-mesh sieve.

Three experiments were carried out. The first involved two groups of 20 rats each, both were fed cariogenic diet 18, but one group was used to establish the restricted regimen described below. The details of this pilot experiment are not reported here, but there was such an unexpected reduction in dental caries from an average of 6.10 carious teeth and 9.35 carious areas per rat for those fed the cariogenic diet ad libitum, to 0.95 carious teeth and 1.0 carious area per rat for those on the restricted regimen that additional work was planned and reported here as experiments 1 and 2. All rats were weaned at three weeks of age and maintained in individual cages for the experimental period of 100 days.

The diets, the experimental period, treatment of the jaws, and the technique of examining and scoring the teeth were the same as reported previously (Miller and Schlack, '58; Miller, '58).

Experiment 1. Eight litters of rats were divided into three littermate groups of 20 each. The rats had access at all times to two of the diets; diet 15, our stock diet, referred to as a semi-natural diet (group 1), and diet 18 in which 17% of the whole wheat flour of diet 15 was replaced with powdered sucrose containing 3% of cornstarch (group 2). For diet 18R (group 3), the same diet 18 was used but the time the rats had access to the food was greatly limited by a plan applied uniformly to all rats in this group. The ingredients of all the diets were fine enough to pass through a 48-mesh sieve.

The restricted feeding regimen was as follows. The first week after weaning, diet 18 was fed ad libitum day and night for three days; then for two days the diet was available overnight but not all day; and for the next two days the rats were fed two times a day but not at night. Beginning with the second week and continuing to the end of the experiment, 4 days of the week (Tuesday, Wednesday, Thursday, and Friday), the rats were fed two times a day, two hours in the morning from about 8 to 10 A.M., and two hours in the afternoon from 2 to 4 P.M. On Saturday they were fed for one hour in the morning (8 to 9 A.M.), after which the food cups were removed. From 12 noon Saturday until Monday at 8 A.M., the rats had access to the food at all times. On Monday morning, food cups were removed from the cages and again replaced from 1 to 3 P.M. It was found necessary to modify the more restricted regimen as indicated above for the first week in order to have the rats thrive and grow normally. It was thus possible, after the first week, to restrict rats in group 3 to 63 hours per week when they could eat the diet as compared with those fed ad libitum who could, if they desired, eat at frequent intervals for 168 hours per week.

Experiment 2. As 15 litters were needed for this experiment, and our small breeding colony does not produce this number at one time, the young were started on the experimental regimen as they became available. Each litter was divided among 6 groups fed as follows: group 1, diet 15, ad libitum; group 2, diet 18, ad libitum; group 3, diet 18R, same as diet 18 but restricted for feeding time as described above; group 4, diet 18R with a supplement of cookies (commercial vanilla wafers); group 5, diet 18R, with a supplement of cooked green soybeans;[4] group 6, diet 18R with a supplement of fresh whole milk. The quantities of the supplements were planned to contribute the approximate isocaloric values of the basal diet that the rats missed by not having access to the basal diet at all times. The rats usually ate the supplements at once, especially the cookies, although some took a longer time to consume the milk and soybeans. The supplements were relatively small. For example, the male rats were given 3 gm of cookies or 16 ml of milk daily, divided between morning and after-

[4] Children of Oriental ancestry in Hawaii sometimes eat green soybeans, boiled in the pods, between meals. They pop the cooked soybeans from the pods into their mouths — a good health habit compared with the constant between-meal eating of sweet and sticky products.

noon feedings for the first half of the experiment, and 4 gm of cookies or 22 ml milk for the remainder of the experimental period of 100 days. For all female rats the amounts were less because of their smaller size and lower food consumption. These supplements were fed two times a day when the food cups containing the basal diets were removed from the cages. The quantities of the supplements the rats would have had if the regimen for Monday, Saturday, and Sunday could have been the same as for Tuesday, Wednesday, Thursday, and Friday were divided between Monday and Saturday, so that by Saturday noon, if the rats had not completed their supplements by the time the food cups were replaced, they had both the basal diet and the supplement. The aim was to learn whether the type of food used for the supplements would influence dental decay, much as one might expect between-meal eating to influence oral conditions in the mouths of children.

Prior to subjecting the data to evaluation by analysis of variance the original observations were, in all cases, subjected to transformation (Snedecor, '57, pp. 314–321). The specific transformations used were $(n + 3/8)^{1/2}$ for the number (n) of defective teeth and for the numbers of carious areas, and the $\log_{10}(s + 1)$ for the caries scores (s).

RESULTS AND DISCUSSION

The results for experiment 1 are summarized in tables 1 and 2, and for experiment 2 in tables 3 and 4. For both experiments, the rats fed the restricted diets consumed about 80% as much food as the unrestricted rats, which is considerably more than the 60 and 40% that has been used by other investigators. Although the rats fed the restricted diet did not weigh as much as unrestricted rats, they appeared healthy and in good condition when killed. A study of their growth, weight, body length, and visceral fat will be the subject of another paper and will not be discussed here except to note that for both experiments 1 and 2 the restricted rats ate about 80% as much as the unrestricted, and their terminal weights were also about 80% of the unrestricted (tables 1 and 3).

As in previous experiments, the teeth of rats fed diet 15 were in much better condition than those fed diet 18 ad libitum (tables 2 and 4), there being a statistically significant difference at the 1% level for the usual three criteria.

As observed for the pilot experiment, a great reduction in dental caries resulted when the rats had restricted access to the cariogenic diet and were without food for 5 nights, which is the natural feeding time for these nocturnal animals. There were significantly lower values ($P < 0.01$, tables

TABLE 1

Summary of results of feeding a semi-natural diet, and a cariogenic diet ad libitum and restricted

Diets	15	18	18R (restricted)
No. of rats	20	20	20
Rats with caries	16	20	9
Rats with caries, %	80	100	45
Carious teeth, total no.	46	141	18
Carious teeth per rat	2.30	7.05	0.90
Carious areas, total	61	228	18
Carious areas per rat	3.05	11.40	0.90
Caries score, total	87	358	18
Caries score per rat	4.35	17.90	0.90
Mean weight, males, gm	342	356	288
Mean weight, females, gm	228	236	195
Average food consumption per rat for 100 days, gm			
Males		1585	1260
Females		1235	991

TABLE 2

Transformed (SR and L) means, successive differences, rectified treatment means, and differences required for significance (H.S.D.) (exp. 1)

Group	Diet	Teeth			Carious areas			Scores		
		SR	Δ[1]	N′	SR	Δ	N′	L	Δ	S′
3	18R	1.014		0.65	1.014		0.65	0.203		0.60
1	15	1.516	0.502	1.92	1.672	0.658	2.42	0.564	0.361	2.66
2	18	2.698	1.182	6.90	3.376	1.704	11.02	1.229	0.665	15.94
H.S.D.[2] ($P < 0.01$)			0.499			0.637			0.282	

[1] Successive mean differences.
[2] Highly significant difference.

TABLE 3

Summary of results of feeding a semi-natural diet and a cariogenic diet ad libitum, and a cariogenic diet restricted, with and without supplements (exp. 2)

Diets	15	18	18R	18R + green soybeans	18R + cookies	18R + whole milk
No. of rats	20	20	20	20	20	20
Rats with caries	12	20	7	4	12	7
Rats with caries, %	60	100	35	20	60	35
Carious teeth, total no.	23	123	14	8	42	11
Carious teeth per rat	1.15	6.15	0.70	0.40	2.10	0.55
Carious areas, total	26	204	15	8	50	11
Carious areas per rat	1.30	10.2	0.75	0.40	25.0	0.55
Caries scores, total	29	329	17	8	57	12
Caries score per rat	1.45	16.45	0.85	0.40	2.85	0.6
Mean weight, males, gm	331	335	266	295	275	283
Mean weight, females, gm	215	229	185	193	185	197
Average food consumption per rat for 100 days, gm						
Males		1493	1162			
Females		1199	928			

TABLE 4

Transformed (SR and L) means, successive differences, rectified treatment means, and differences required for significance (H.S.D.) (exp. 2)

Group	Diet	Teeth			Carious areas			Scores		
		SR	Δ[1]	N′	SR	Δ	N′	L	Δ	S′
5	18R + green soybeans	0.789		0.25	0.788		0.25	0.089		0.23
6	18R + milk	0.873	0.084	0.39	0.873	0.085	0.39	0.138	0.049	0.37
3	18R	0.921	0.048	0.47	0.931	0.058	0.49	0.164	0.026	0.46
1	15	1.137	0.216	0.92	1.171	0.240	1.00	0.290	0.126	0.95
4	18R + cookies	1.373	0.236	1.51	1.434	0.263	1.68	0.390	0.100	1.46
2	18	2.531	1.158	6.03	3.204	1.770	9.89	1.194	0.804	14.63
H.S.D. $P < 0.01$			0.555			0.643			0.297	
$P < 0.05$			0.457			0.545			0.248	

[1] Successive mean differences.

2 and 4) for dental caries as judged by the three criteria for both experiments 1 and 2. In fact, for experiment 1, the rats on the 18R regimen showed such a great improvement in the condition of their teeth that the data for teeth, areas, and scores showed a highly significant difference from those of the rats receiving diet 15 (table 2). For experiment 2, although the caries rate was low for the restricted group, there was not a statistically significant reduction over those receiving diet 15 (table 4).

When the supplement to the restricted diet was cookies, decay was greater than for the restricted diet alone; also more decay was noted when cookies were given than when the supplements were green soybeans or milk, most of the differences being significant at $P < 0.01$ (table 4). There were some improvements in the condition of the teeth when the supplements were green soybeans or milk as compared with the restricted diet alone, but these did not prove to be statistically significant (tables 3 and 4).

These results support the idea that limiting the time a cariogenic diet is in contact with the mouth will markedly influence the extent and severity of dental caries. They also suggest, since the nature of the "between-meal snacks" was of great importance in promoting or allaying dental decay in rats, that this will also be true for humans. Apparently, it is not so much the *amount* of sugar in the diet, providing the nutrients essential for an adequate diet are ingested, as the *frequency* of eating sweets and other carbohydrate foods which promotes oral conditions conducive to dental decay.

SUMMARY

When susceptible rats were allowed access to a highly cariogenic diet according to a regimen established by a pilot experiment, there was a highly significant statistical reduction in dental caries as judged by three criteria — the number of decayed teeth, the number of carious areas and the caries score — compared with the littermate rats fed ad libitum. The restricted rats ate about 80% as much food as the unrestricted rats.

In one experiment those on restricted feeding had better teeth than littermate rats fed a semi-natural diet; in the second experiment, the differences were not statistically significant.

Three groups of rats on the restricted regimen were given supplements of cookies, cooked green soybeans, or whole fresh milk during the morning and afternoon hours when the food cups were removed from the cages. The group given cookies had poorer teeth than those given soybeans or milk ($P < 0.01$). Although the group fed cookies had significantly more caries than the restricted group, there was not sufficient improvement in the teeth of those fed the milk or soybeans to be statistically significant compared with the group fed the cariogenic diet on a restricted schedule.

These experiments support those of others in proving that the hours the teeth are in contact with a cariogenic diet is of prime importance in promoting or allaying dental decay.

ACKNOWLEDGMENTS

The author gratefully acknowledges the work of J. G. Darroch in recommending and checking the statistical treatment of the results, and the technical assistance of Mrs. Mildred Ige.

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Effect of Antioxidants and Unsaturated Fatty Acids on Reproduction in the Hen

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Unsaturated fatty acids of the linoleic acid family in the diet increase the requirement of the chick for vitamin E or other antioxidants causing encephalomalacia (Dam et al., '58; Century et al., '59; Machlin and Gordon, '60). Studies with humans (Horwitt, '60) and the rat (Century and Horwitt, '60) also indicate that the vitamin E requirements are increased when unsaturated fatty acids are fed.

Adamstone and Card ('34) observed that feeding of vitamin E-deficient diets for two years resulted in testicular degeneration in some of the deficient male chickens. However, there is no evidence with poultry that a deficiency of this vitamin will result in sterility of the female. Studies with females (Card et al., '30; Singsen et al., '54; Jensen et al., '53) indicated that vitamin E had no effect on fertility or on egg production although it was essential for maintenance of hatchability.

The object of the following studies was to determine whether hens fed high linoleic acid diets require vitamin E or an antioxidant or both for maintenance of normal fertility, hatchability, and egg production and whether the requirement for antioxidants for these functions is related to the linoleic acid level of the diet.

EXPERIMENTAL

The composition of the purified basal diets is shown in table 1. Alkaline-refined safflower oil was used as a source of linoleic acid. Most of the tocopherol was destroyed by refluxing the oil in an acetone solution in the presence of ferric chloride. The acetone, ferrous and ferric chlorides were then removed from the oil by extensive washing with a dilute Versene Fe-specific solution and finally with water.

TABLE 1
Composition of experimental diets

Ingredient	Diet	
	S-25-A	S-25-B
	%	%
Isolated soybean protein[1]	25.00	25.00
Treated safflower oil	10.00[2]	10.00[3]
Cellulose[4]	10.00	8.00
Methionine hydroxy analogue[5]	0.30	0.40
Vitamin A (10,000 IU/gm)	0.20	0.20
Vitamin D_3 (7,500 ICU/gm)	0.10	0.05
Vitamin mixture S-35[6]	0.50	0.60
Choline (25% mix)	0.50	1.00
Mineral mixture:		
$CaCO_3$	2.80	2.80
$Ca_3(PO_4)_2$	2.80	2.80
K_2HPO_4	0.90	1.00
$MgSO_4 \cdot 7H_2O$	0.25	0.25
$Fe(C_6H_5O_7) \cdot 6H_2O$	0.14	0.14
$Zn\ CO_3$	0.002	0.015
KI	0.004	0.004
$CuSO_4 \cdot 5H_2O$	0.002	0.002
H_3BO_3	0.0009	0.0009
$CoSO_4 \cdot 7H_2O$	0.0001	0.0001
$MnSO_4$	0.065	0.065
NaCl	0.880	0.700
$Na_2SeO_3 \cdot 5H_2O$	0.0001	0.0006
Corn starch	0.0549	0.0224
Glucose[7]	45.50	46.95

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Cincinnati.
[2] Treated with ferric chloride to destroy tocopherol.
[3] Air oxidized 32 hours, 72°C to destroy tocopherol.
[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[5] Registered trademark of the Monsanto Chemical Company for calcium DL-2-hydroxy,4-methylthiobutyrate.
[6] When added at 0.5% of the diet this supplied in mg/kg of finished diets; vitamin B_{12}, 0.030; biotin, 0.30; menadione, 1.0; pyridoxine·HCl, 8.0; folic acid, 4.0; riboflavin, 16.0; Ca pantothenate, 20.0; thiamine·HCl, 24.0; nicotinic acid, 100.0.
[7] Cerelose, Corn Products Company, New York.

This process reduced the α-tocopherol level from 370 mg per pound of oil to an average of 50 mg per pound when assayed by the procedure of Bro-Rasmussen and Hjarde ('57). In the second experiment the tocopherol was destroyed by

Received for publication July 29, 1961.

TABLE 2

α-Tocopherol and linoleic acid content of dietary fats

	α-Tocopherol	Linoleic acid
	mg/pound	%
Safflower oil[1] treated with ferric chloride	50	70.0
Air-oxidized safflower oil[1]	11	72.0
Tallow[2]	12	5.5
Hydrogenated coconut oil[3]	7	1.0

[1] Alkaline-refined safflower oil from the Pacific Vegetable Oil Corporation, San Francisco.
[2] Number 1 prime beef tallow.
[3] The Blanton Company, St. Louis, Missouri.

passing air through the safflower oil for 32 hours at 72°C. In each instance 0.1% of an antioxidant mixture[1] was added after the treatments to minimize subsequent development of rancidity. All fats were analyzed for α-tocopherol (Bro-Rasmussen and Hjarde, '57) and for linoleic acid (Marco et al., '61). These values are shown in table 2.

White Leghorn pullets,[2] 25 weeks old, that had been maintained with commercial growing and laying diets prior to the experiment were used. The birds were kept in individual cages with individual waterers and fed in units of 4 birds each. All pullets were inseminated once weekly with 0.1 ml of semen obtained by mixing semen from three to four cockerels fed commercial breeding rations. Eggs were placed in the incubator every two weeks. Eggs collected during the 10-day period prior to the setting were used for the hatchability studies. All eggs that appeared infertile at the tenth day by candling were broken and examined for apparent fertility. The experimental designs are shown in table 3.

RESULTS AND DISCUSSION

In contrast with previous reports (Card et al., '30; Singsen et al., '54; Jensen et al., '53), egg production of hens fed the diet low in vitamin E decreased rapidly compared with that of groups fed either vitamin E or ethoxyquin[3] (fig. 1). The low egg production was apparently a result of the high linoleic acid content of this diet, since hens that were changed from the basal diet (7.0% linoleic acid) to a diet containing tallow (0.5% linoleic acid) increased in production until they were laying as well as those groups fed antioxidants. Addition of 20 or 100 IU of vitamin E per pound to the basal diet restored egg production to control levels, whereas addition of 0.075% of ethoxyquin merely prevented a further decline in production.

The inability of the antioxidant to restore egg production (or other functions) could be interpreted in two ways. (1) The antioxidant is effective by protecting tissue stores of vitamin E and therefore when these stores are depleted it is no longer effective. (2) The requirement for

[1] Tenox 6. This contains 10% of butylated hydroxytoluene (BHT) and 10% of butylated hydroxyanisole (BHA), available from Eastman Chemicals Products, Inc., Kingsport, Tennessee.
[2] A light commercial hybrid, H and N "nick" chick developed by the Heisdorf and Nelson Company, Kirkland, Washington.
[3] As Santoquin, registered trademark of the Monsanto Chemical Company, for 1,2-dihydro-6-ethoxy 2,2,4 trimethylquinoline.

TABLE 3

Experimental design

Experiment 1			Experiment 2	
Supplement 1 to basal diet S-25-A (0–8 weeks)	Supplement 2 to basal diet S-25-A (9–16 weeks)	No. hens/ treatment	Supplement to basal diet S-25-B (0–6 weeks)	No. hens/ treatment
None	None	8	None	40
None	Vitamin E,[1] 20 IU/lb.	8	Vitamin E, 100 IU/lb.	8
None	Vitamin E, 100 IU/lb.	8	Ethoxyquin, 0.075%	8
None	Ethoxyquin,[2] 0.075%	8	Ethoxyquin, 0.300%	8
None	Tallow, 10%	8	Hydrogenated coconut oil,[2] 10%	8
Vitamin E, 100 IU/lb.	None	8	Hydrogenated coconut oil,[2] 9%, + peroxidized safflower oil, 1%	8
Ethoxyquin, 0.075%	None	8		
Vitamin E, 100 IU/lb. + ethoxyquin, 0.075%	Vitamin E, 100 IU/lb. + ethoxyquin, 0.075%			

[1] Added as Myvamix, a concentrate containing 20,000 IU of vitamin E per pound, obtainable from Distillation Products Industries, Rochester, New York.
[2] Replaces treated safflower oil.

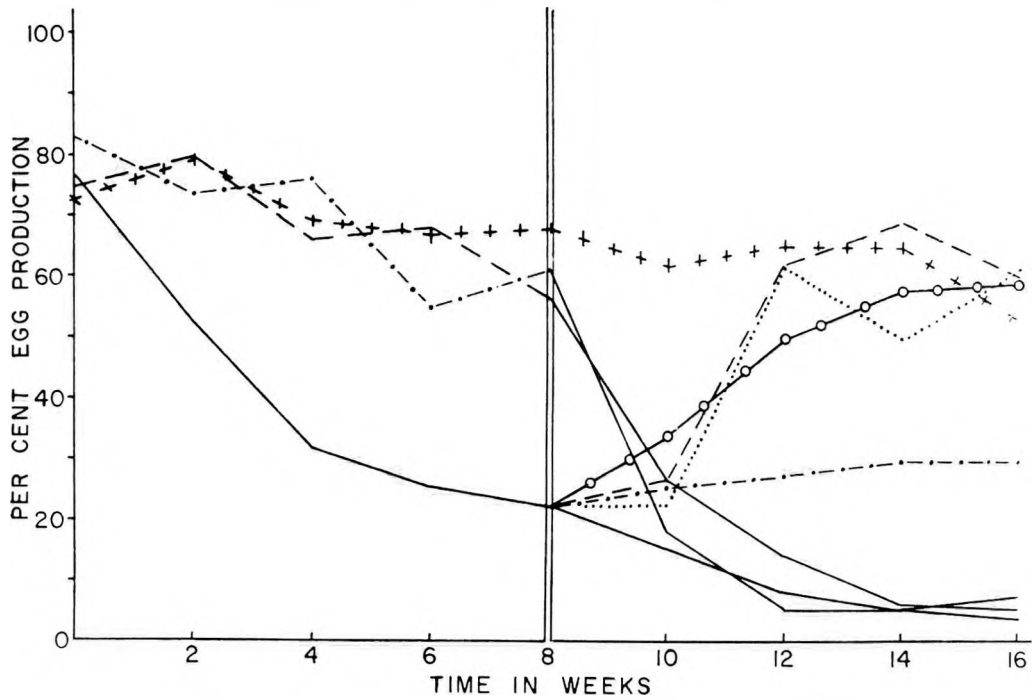

Fig. 1 Experiment 1, egg production. Key: ———, basal; ·—·—·, 0.075% of ethoxyquin; ○——○, 20 IU of vitamin E/pound; — — —, 100 IU of vitamin E/pound; + + +, ethoxyquin plus vitamin E; . . . . . , 10% of tallow.

an antioxidant for restoration of egg production (and other functions) is higher than that needed for maintenance. A higher requirement would be predicted if *in vivo* autoxidation is occurring since autoxidation is an autocatalytic reaction and less antioxidant would be required to inhibit the initiation of oxidation than after oxidation has proceeded for some time.

It is possible that the decline in egg production is a result of an *in vivo* autoxidation of lipid-amino acid complexes in the oviduct. Hendler ('59) has postulated that these lipid-amino acid complexes are intermediate carriers for amino acids during protein synthesis in the oviduct. The lipid-amino acid bonds would be expected to be susceptible to autoxidation of the lipid moiety (Nishida, '60) and disruption of the complex could then interfere with protein synthesis.

The second experiment was complicated by a nonspecific toxic effect of the air-oxidized safflower oil. Although oxidation conditions were mild and the peroxide number of the oxidized oil was low, feeding of this oil to young chickens resulted in rickets and growth reduction. The toxicity of the air-oxidized oil might account for the relatively poor egg production in groups fed the high linoleic acid diet in experiment 2, with or without the addi- of vitamin E or ethoxyquin (fig. 2).

Fertility. It has not been reported previously that antioxidants or fatty acids, or both, can influence fertility of the female chicken. Since histological examinations of the germinal disk were not made in these studies, it is possible that a portion of eggs classified infertile represented early embryonic death. In both experiments (figs. 3 and 4) apparent fertility decreased to about 30% in groups fed the basal diet compared with approximately 60% when the diet was supplemented with either vitamin E or ethoxyquin. Fertility was high in groups fed 0.7% of linoleic acid (fig. 4, 9% of hydrogenated coconut oil plus 1% of safflower oil treat-

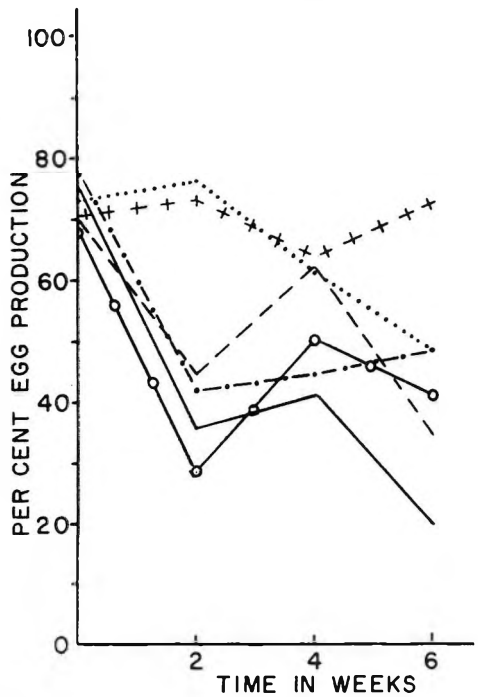

Fig. 2 Experiment 2, egg production. Key: ———, basal; ·—·—·, 0.075% of ethoxyquin; ○——○, 0.300% of ethoxyquin; — — —, 100 IU of vitamin E/pound; . . . . . , 10% of hydrogenated coconut oil; + + +, 9% of hydrogenated coconut oil plus 1% of peroxidized safflower oil.

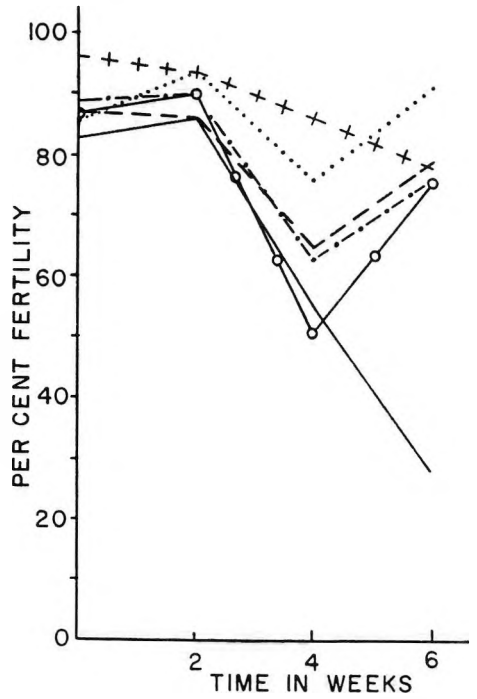

Fig. 3 Experiment 2, fertility determined by microscopic examination after 10 days of incubation. Key: ———, basal; ·—·—·, 0.075% of ethoxyquin; ○——○, 0.300% of ethoxyquin; — — —, 100 IU of vitamin E/pound; . . . . . , 10% of hydrogenated coconut oil; + + +, 9% of hydrogenated coconut oil plus 1% of peroxidized safflower oil.

ment) compared with that in groups fed 7.2% of linoleic acid.

In experiment 1 addition of ethoxyquin to the basal diet at 8 weeks only partially restored fertility to levels comparable to that observed in birds fed vitamin E. This observation could be interpreted in the same manner as restoration of egg production.

Substitution of tallow for safflower oil also only partially restored fertility. The addition of safflower oil in the first period would increase linoleic acid in both the depot fat and eggs (Feigenbaum and Fisher, '59; Wheeler et al., '59). It is not known, however, how rapidly the linoleic acid would decrease when a tallow-containing diet is fed. It is possible that the linoleic acid level is sufficient after 8 weeks to influence fertility adversely.

Hatchability. The effect of feeding the linoleic acid diet on hatchability was dramatic, decreasing from 77% to zero within 6 weeks (fig. 5). In experiment 1 this profound decrease was prevented by the addition of 100 IU of vitamin E per pound of ration but not by 0.075% of ethoxyquin. Evidently this level of ethoxyquin is insufficient since in experiment 2, higher levels of ethoxyquin (0.30%) prevented the decline in hatchability (fig. 6). The requirement for vitamin E is also quite high since in the second period of experiment 1 (fig. 5) 20 IU of vitamin E per pound of ration had almost no effect on hatchability. Substituting beef tallow for safflower oil only partially restored hatchability.

If it is assumed that the requirement for maintenance of hatchability with the high linoleic acid diet is 100 IU of vitamin E per pound of ration (0.022% of *dl*-α-tocopheryl acetate) or 0.30% of ethoxyquin, then dietary vitamin E is approximately 14 times more effective on a weight basis than ethoxyquin for this

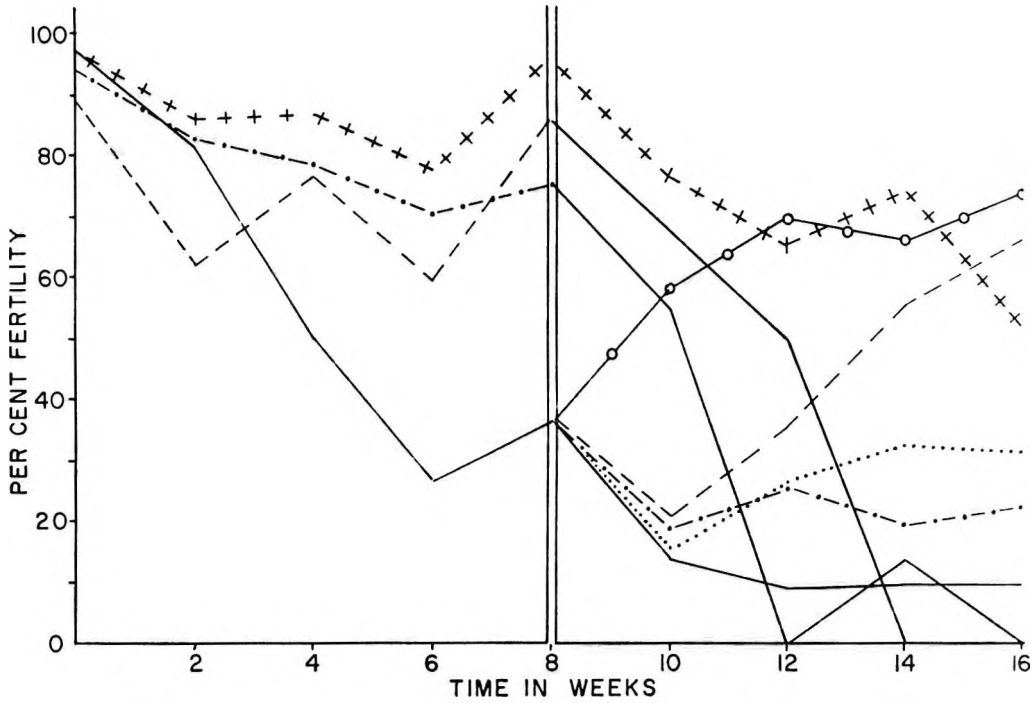

Fig. 4 Experiment 1, fertility determined by microscopic examination after 10 days of incubation. Key: ———, basal; ·—·—·, 0.075% of ethoxyquin; ○—○, 20 IU of vitamin E/pound; — — —, 100 IU of vitamin E/pound; + + +, ethoxyquin plus vitamin E; . . . . . , 10% of tallow.

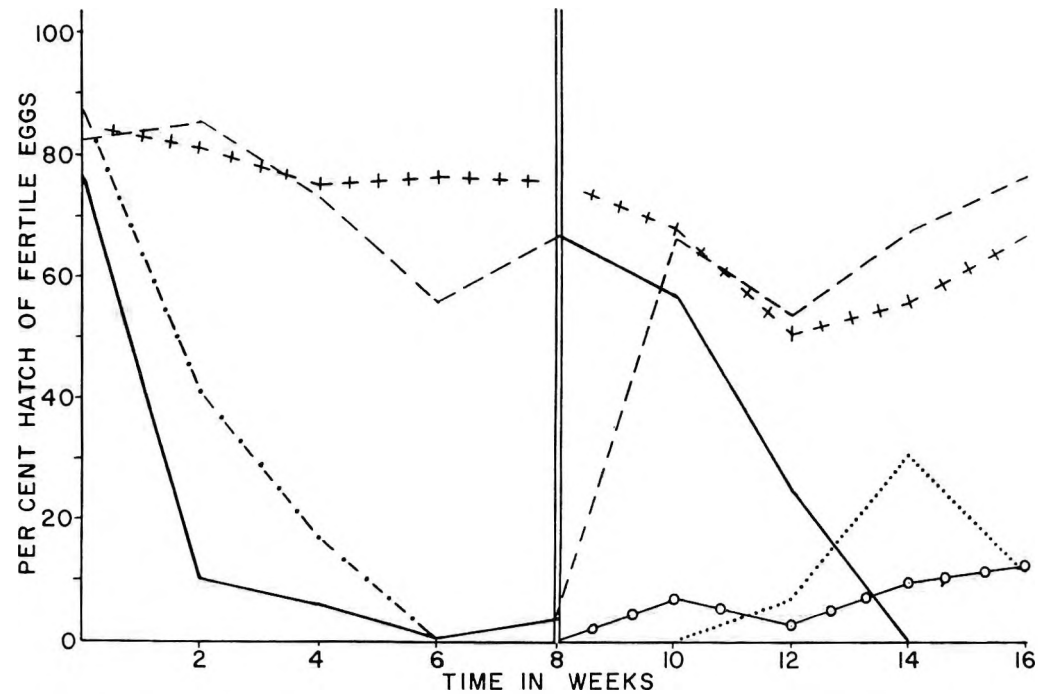

Fig. 5 Hatch of fertile eggs. Key: ———, basal; .—.—., 0.075% of ethoxyquin; ○—○, 20 IU of vitamin E/pound; — — —, 100 IU of vitamin E/pound; + + +, ethoxyquin plus vitamin E; . . . . . , 10% of tallow.

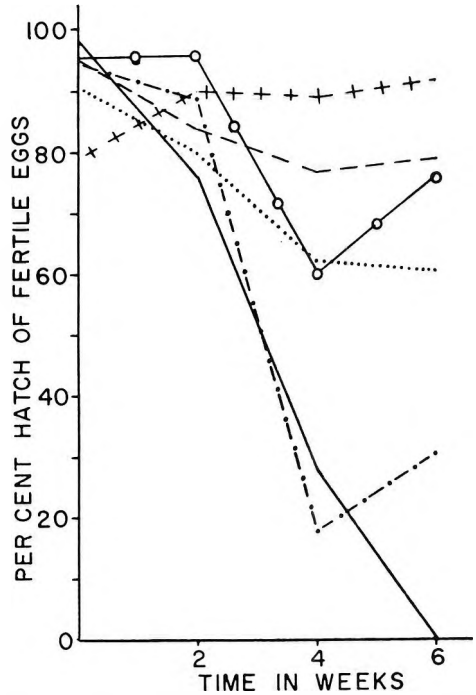

Fig. 6 Experiment 2, hatch of fertile eggs. Key: ———, basal; ·—·—·, 0.075% of ethoxyquin; ○—○, 0.300% of ethoxyquin; — — —, 100 IU of vitamin E/pound; . . . . . , 10% of hydrogenated coconut oil; + + +, 9% of hydrogenated coconut oil plus 1% of peroxidized safflower oil.

function. Over 99% of dietary ethoxyquin is excreted within 24 hours and only minute amounts are deposited in the egg (Machlin et al., '57). This contrasts with the ability of the hen to deposit considerable (16% of the dietary intake) dietary vitamin E in the egg (Dju et al., '50). Based on the data of Dju et al. ('50), one would estimate that eggs laid by hens fed diets with 0.022% of vitamin E per pound would contain approximately 40 ppm of α-tocopherol. Only 4 to 8 ppm of ethoxyquin would be deposited when hens are fed 0.30% ethoxyquin. This suggests that ethoxyquin may be more effective than α-tocopherol once it is deposited in the egg, and correlates well with the comparative antioxidant properties of these two compounds (Machlin et al., '59). The study also emphasizes that the high biological activity of α-tocopherol may be a consequence of its greater availability for tissue deposition compared with other antioxidants.

SUMMARY

Hens were fed a purified diet, low in antioxidants and high in linoleic acid for 8 weeks. By the end of this period egg production of the hens had decreased from 78 to 25%; only 37% of the eggs were fertile and none of the fertile eggs hatched. At the end of 8 weeks, hens fed the basal diet supplemented with 100 IU of vitamin E per pound, had an average egg production of 57%; 76% of their eggs were fertile and 67% of the fertile eggs hatched. When the diet was supplemented with 0.075% of ethoxyquin, egg production was 61%; 76% of the eggs were fertile but only 4% of the fertile eggs hatched. Addition of ethoxyquin at a higher level (0.3%) resulted in hatchability comparable to that of vitamin E-supplemented groups. In addition it was noted that with low linoleic acid diets there was no requirement for vitamin E or antioxidants for maintenance of egg production, fertility and hatch of fertile eggs. Apparently the hen responds to the same dietary stresses that produce encephalomalacia in chicks by exhibiting a reduction of egg production, fertility, and hatchability. These dietary stresses can be overcome by increasing dietary levels of antioxidants of either natural or synthetic origin or by decreasing the linoleic acid content of the diet.

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The Post-Cold Behavior of Riboflavin-Deficient Rats[1]

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In a study of a pantothenic acid deficiency induced in a cold (5°C) environment (Vaughan and Vaughan, '60), it was observed that removal of the cold animals to a warm room was followed by a spurt in growth unrelated to the level of the vitamin in the diet and far exceeding the growth of warm rats at the same stage of deficiency. This effect was attributed in part to an elevation in food intake which persisted for as long as three weeks after the rats were removed from the cold room.

In the case of riboflavin, elevated liver levels have been reported in cold-exposed animals (Fontaine and Raffy, '42). Bessey et al. ('58), furthermore, observed no evidence of increased riboflavin disappearance in cold-exposed rats and data have been presented (Vaughan and Vaughan, '59) to indicate that the requirement for riboflavin in rats, when expressed in terms of food intake, is not increased in a cold environment.

In the experiments reported here, we studied a riboflavin deficiency to determine whether the imposition of cold exposure during depletion would affect the course of the deficiency after removal from the cold environment, as it does in the case of a pantothenic acid deficiency.

EXPERIMENTAL

Experiment 1. Thirty male Sprague-Dawley rats, weighing 240 to 270 gm were fed a riboflavin-deficient diet[2] for 4 weeks. During this time, 15 of these rats were kept in a cold (5°C) room in screen-bottom cages, while the remaining 15 were maintained at 25°C. At the end of the 4-week period, each group of 15 rats was divided into three sub-groups of 5 rats each; one sub-group continued to be fed the riboflavin-deficient diet, while the other two received a supplement of 1.0 μg of riboflavin per gm of diet and 5.0 μg of riboflavin per gm of diet. At this time the 15 cold rats were transferred to the warm room and the experiment was continued for 7 weeks. During the full 11-week period, food intakes and body weights were measured.

Experiment 2. Ninety male Sprague-Dawley rats weighing 145 to 180 gm were assigned a riboflavin-deficient diet.[2] Forty-five of these rats were placed in a cold room at 5°C; the remaining 45 were kept at 25°C. The cold rats were exposed for 4 weeks; at the end of this time, they were returned to the warm room[3] and continued to be fed the riboflavin-deficient diet for 5 additional weeks. The riboflavin-deficient warm rats remained at 25°C for the whole 9-week period. At the beginning of the fifth week, the diet of one-half of the warm group and one-half of the post-cold group was supplemented with riboflavin at the level of 5 μg per gm of diet. At the end of the sixth week, the warm rats receiving this supplement were placed in the cold (5°C) room and remained there until the end of the ninth week. Food intakes and body weights were measured throughout the experiment.

At the end of the second, fourth, fifth, sixth, and ninth week, 5 rats from each experimental group were sacrificed and analyzed for the following: liver riboflavin, liver xanthine oxidase, kidney xanthine oxidase, and total carcass (without intestinal contents and liver) fat, protein, and water. As initial controls, 5 stock diet-fed rats from the same group were analyzed at the beginning of the experiment.

Received for publication July 10, 1961.

[1] The views expressed are those of the authors and do not necessarily represent official Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association. (AFR 160-94).

[2] Purchased from Nutritional Biochemicals Corporation. Cleveland. Ingredients are listed in Vaughan and Vaughan ('59).

[3] Thereafter referred to as "post-cold" rats.

Liver riboflavin was determined using the extraction procedures of Bessey et al. ('58) and the fluorometric method of McLaren et al. ('44). Liver and kidney xanthine oxidase activity was measured by the method of Dietrich and Borries ('54), and is expressed as micromoles of xanthine converted to allantoin (liver) or uric acid (kidney). Carcass fat was determined by ether extraction of the dried, ground material and carcass protein by the microKjeldahl method.

RESULTS

Gross symptoms. In figure 1 is illustrated the difference in appearance in rats after 10 weeks of receiving the riboflavin-deficient diet between those that spent the entire 10 weeks at 25°C and rats that spent the initial 4 weeks at 5°C, followed by 6 weeks at 25°C. The 25° rat exhibited the striking "drowned rat" appearance of a riboflavin deficiency, whereas the post-cold rat was relatively free of these symptoms. These rats were photographed during experiment 1.

Weight changes. Growth patterns of the deficient rats in both experiments are shown in figure 2. When fed the riboflavin-deficient diet, the warm rats continued to grow for about three weeks, after which growth ceased and they began to lose weight slowly. The cold rats, on the other hand, ceased growing immediately in experiment 1, and at the end of three weeks in experiment 2. When these cold rats were returned to the warm room, however, they began to grow slowly while at the same time the warm rats continued to lose weight. At the end of both experiments, the weights of the post-cold rats were slightly greater than those of the warm rats as a result of this slow post-cold growth. In experiment 1, final weights at the end of 11 weeks were: warm, 242 ± 16 gm; post-cold, 249 ± 9 gm. Weight changes from the fourth to eleventh week were: warm, − 28 ± 10; post-cold, + 18 ± 8. In experiment 2, final weights of the deficient animals at the end of 9 weeks were: warm, 176 ± 5; post-cold, 178 ± 11. Weight changes from the fourth to ninth week were: warm, − 14 ± 8; post-cold, + 17 ± 4.

A comparison of the "stripped weights" (carcass minus intestinal contents and

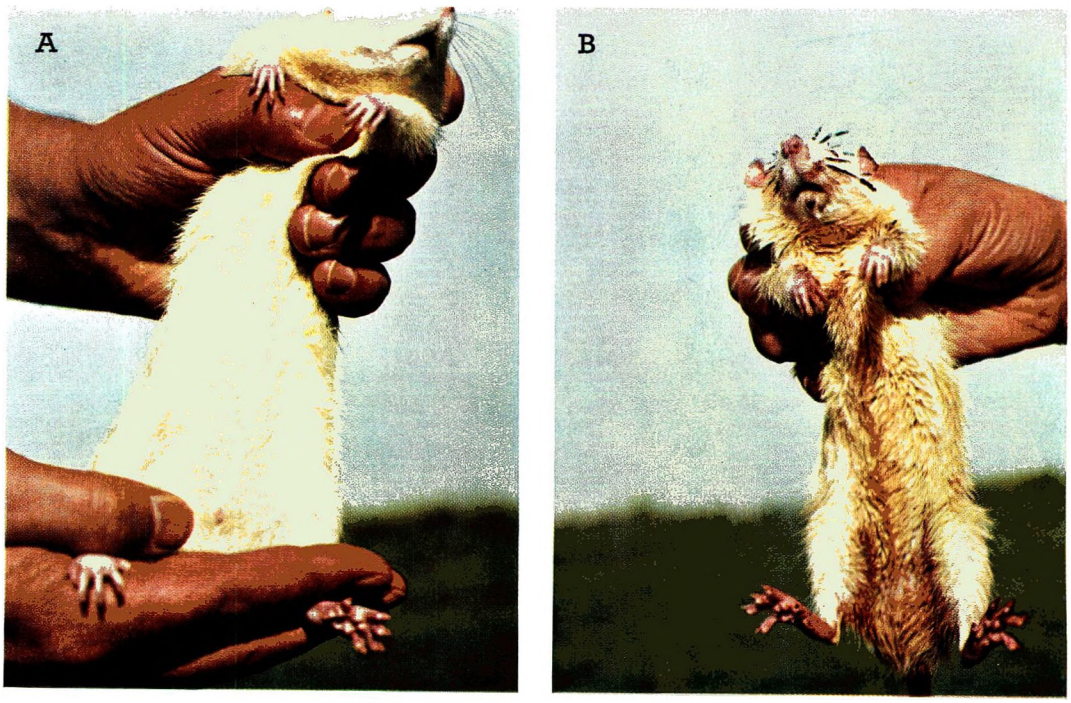

Fig. 1 Riboflavin-deficient rats. A, post-cold; B, warm.

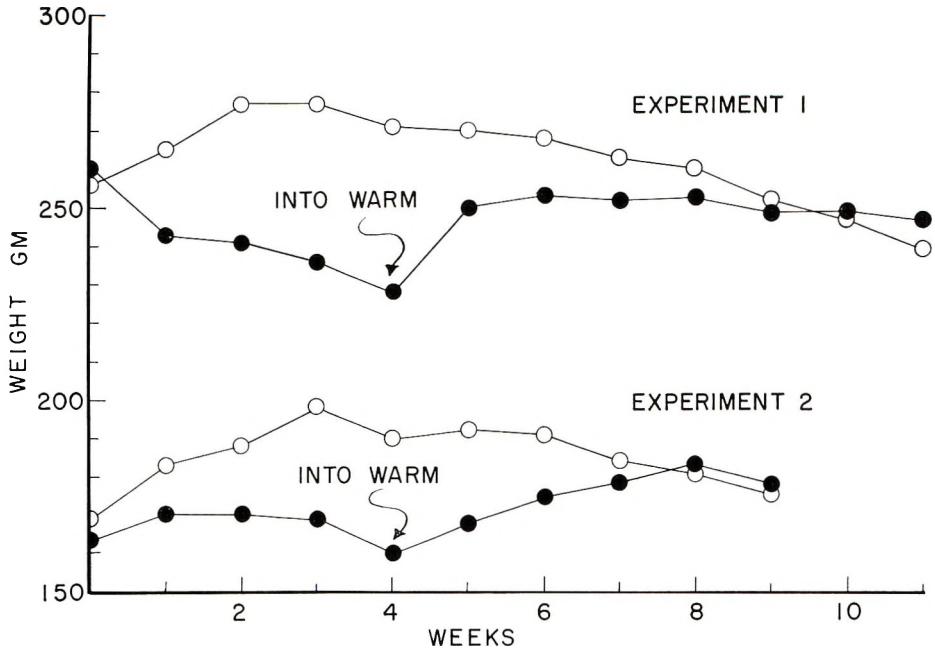

Fig. 2 Growth curves of depleted rats. ○, warm; ●, cold and post-cold; dashed lines, repletion; solid lines, depletion.

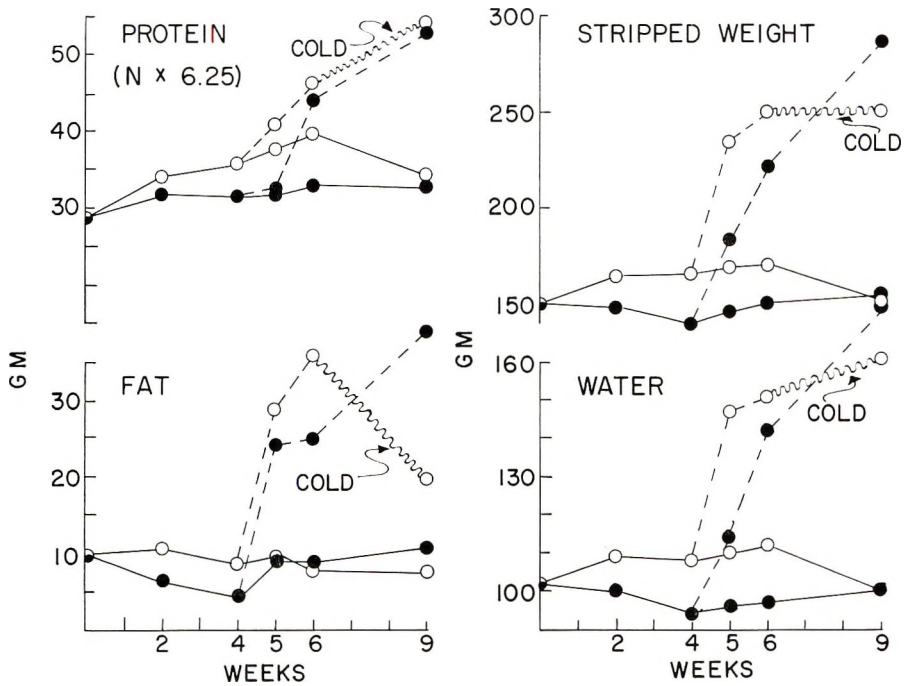

Fig. 3 Carcass composition of experimental animals. ○, warm; ●, cold and post-cold; dashed lines, repletion; solid lines, depletion.

TABLE 1

Fractional weight changes, total liver riboflavin levels and liver and kidney xanthine oxidase activities of experimental animals

Week	Stripped weight (gm)[1]		Water (gm)		Protein (gm)		Fat (gm)		Total liver vitamin B_2 (μg)		Xanthine oxidase (μmole/gm/hour)			
											Liver		Kidney	
	Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold
							Riboflavin-deficient animals							
0	150 ± 3.8[2]		101.6 ± 2.8		28.8 ± 1.4		9.9 ± 1.2		337.6 ± 18.0		1.65 ± 0.07		1.06 ± 0.09	
2	164 ± 6.0	149 ± 4.5	109.3 ± 4.1	100.8 ± 3.1	34.0 ± 1.2	31.7 ± 1.1	10.7 ± 0.85	6.3 ± 0.38	124.0 ± 9.8	143.1 ± 6.0	1.41 ± 0.09	1.44 ± 0.11	0.93 ± 0.09	1.16 ± 0.07
4	165 ± 9.1	140 ± 3.2	108.3 ± 6.4	94.0 ± 2.2	35.6 ± 1.6	31.4 ± 1.1	8.6 ± 0.9	4.3 ± 0.4	96.9 ± 9.0	115.7 ± 6.3	1.40 ± 0.13	1.52 ± 0.20	0.58 ± 0.12	0.77 ± 0.12
		Post-cold		Post-cold		Post-cold		Post-cold		Post-cold		Post-cold		Post-cold
5	169 ± 4.5	146 ± 8.0	110.1 ± 3.4	95.9 ± 5.1	37.4 ± 0.8	31.9 ± 1.9	9.7 ± 0.7	9.1 ± 0.7	89.7 ± 4.7	86.4 ± 3.7	0.88 ± 0.03	1.48 ± 0.29	0.41 ± 0.09	0.78 ± 0.07
6	171 ± 5.9	150 ± 6.7	112.3 ± 3.4	97.0 ± 4.3	39.7 ± 2.9	33.0 ± 0.9	7.7 ± 1.1	8.7 ± 1.4	80.9 ± 7.5	89.4 ± 5.2	0.52 ± 0.12	0.74 ± 0.04	0.47 ± 0.10	0.89 ± 0.13
9	153 ± 10.1	155 ± 3.8	100.3 ± 6.0	100.0 ± 1.8	34.3 ± 2.2	32.7 ± 0.9	7.4 ± 1.9	10.6 ± 1.3	67.6 ± 8.5	73.4 ± 3.8	0.42 ± 0.03	0.78 ± 0.20	0.49 ± 0.06	0.71 ± 0.05
							Riboflavin-repleted animals							
5	235 ± 8.1	184 ± 8.3	147.2 ± 6.1	113.6 ± 4.6	40.7 ± 1.8	32.2 ± 0.6	28.8 ± 1.1	24.0 ± 2.6	271.1 ± 13.2	221.9 ± 27.5	1.72 ± 0.28	2.36 ± 0.27	0.85 ± 0.12	1.15 ± 0.21
6	250 ± 5.4	221 ± 4.7	151.4 ± 4.6	141.6 ± 4.0	46.1 ± 1.3	43.7 ± 2.7	35.8 ± 1.9	25.0 ± 1.5	263.7 ± 17.1	248.2 ± 18.4	1.57 ± 0.05	1.82 ± 0.03	1.39 ± 0.08	1.51 ± 0.10
	Cold		Cold		Cold		Cold		Cold		Cold		Cold	
9	251 ± 8.6	286 ± 5.0	161.3 ± 6.8	174.8 ± 4.4	54.0 ± 2.0	52.8 ± 2.1	19.7 ± 2.7	39.3 ± 2.4	390.2 ± 5.8	324.4 ± 6.9	1.89 ± 0.09	1.66 ± 0.08	1.60 ± 0.09	1.48 ± 0.09

[1] See text.

[2] Standard error of the mean.

liver) of the animals, along with the fractional composition of these weights is shown in figure 3 and table 1. The warm deficient rats gained weight throughout the first 6 weeks of the deficient dietary regimen — between the sixth week and the ninth week, however, tissue weight dropped rapidly. These weight gains appear to be a result of increases in protein and water only, for the fat content remained more or less the same throughout the first 5 weeks. Between the sixth and ninth weeks, however, all components of the body were lost.

The initial losses in the cold rats appear to be composed of fat and water, with some actual increase in protein content. Upon removal to the warm room, fat and water were deposited immediately, whereas protein content remained unchanged. From 5 weeks on, these deficient animals continued to gain water and fat until at 9 weeks stripped weight equaled that of the rats which had never been cold.

The effects of supplementing the diet with 5 μg of riboflavin per gm of diet from the fourth to ninth week are also shown in figure 3 and table 1. Both groups of rats deposited all fractions rapidly during the following two weeks and the post-cold rats continued to do so until the end of the experiment. As noted before, the remaining warm rats were placed in the cold room at the end of the sixth week. After three weeks in the cold, the stripped weight of these rats was the same as that of the warm rats killed at 6 weeks. Fractional analysis of these rats, however, showed that during this cold exposure, water and protein continued to be deposited, whereas fat was lost.

Food intake. Food intake of the cold-exposed rats receiving the riboflavin-deficient diet averaged 40% above that of the warm rats receiving the same diet. Upon removal to the warm room, the intake of the post-cold rats decreased sharply and did not exceed appreciably that of the warm rats for the remainder of the experiment. From the fourth to ninth week, average total food intakes were 315 and 337 gm for the warm and post-cold rats, respectively. It would appear that the food efficiency of the post-cold rats had improved, since they gained 27 gm net over the warm rats on a total increment of 22 gm of food.

Riboflavin and xanthine oxidase levels. Total liver riboflavin values are shown in figure 4 and table 1. In both cold and warm rats, the riboflavin-deficient diet caused a rapid decrease in liver riboflavin by two weeks, followed by a more gradual depletion until the end of the experiment. There was no difference apparent between the two groups, although (since the cold rats were smaller than the warm rats) the available liver riboflavin per total body weight was higher in the cold animals. Repletion with riboflavin caused an immediate increase in liver riboflavin in both groups. When the repleted warm rats were placed in the cold room at 6 weeks, their liver riboflavin levels increased further to a point higher than that of the post-cold rats at 9 weeks.

The sequential events with respect to liver and kidney xanthine oxidase are also shown in figure 4 and table 1. In the liver, xanthine oxidase activity remained at control levels for 4 weeks in the warm, and 5 weeks in the cold animals, despite the rapid drop in riboflavin levels. By 6 weeks, both groups appear to have stabilized at much lower levels, with those of the post-cold rats somewhat higher. In the kidney, the behavior of xanthine oxidase was somewhat different. The values for the cold-exposed rats were somewhat higher than those for the warm rats, albeit not significantly. After the 4-week point, however, when the cold rats were transferred to the warm room, their liver xanthine oxidase activities remained fairly high throughout the next 5 weeks, whereas the activity of xanthine oxidase in the kidneys of the warm rats fell to much lower levels. Upon repletion with riboflavin, the xanthine oxidase levels of both groups rose immediately. No differences were noted in the levels between the repleted rats in the warm room at 9 weeks and those in the cold room.

DISCUSSION

The results of experiment 1, which was a preliminary approach, indicated to us that the removal of riboflavin-deficient rats from a cold to a warm environment produced a response which enabled them to

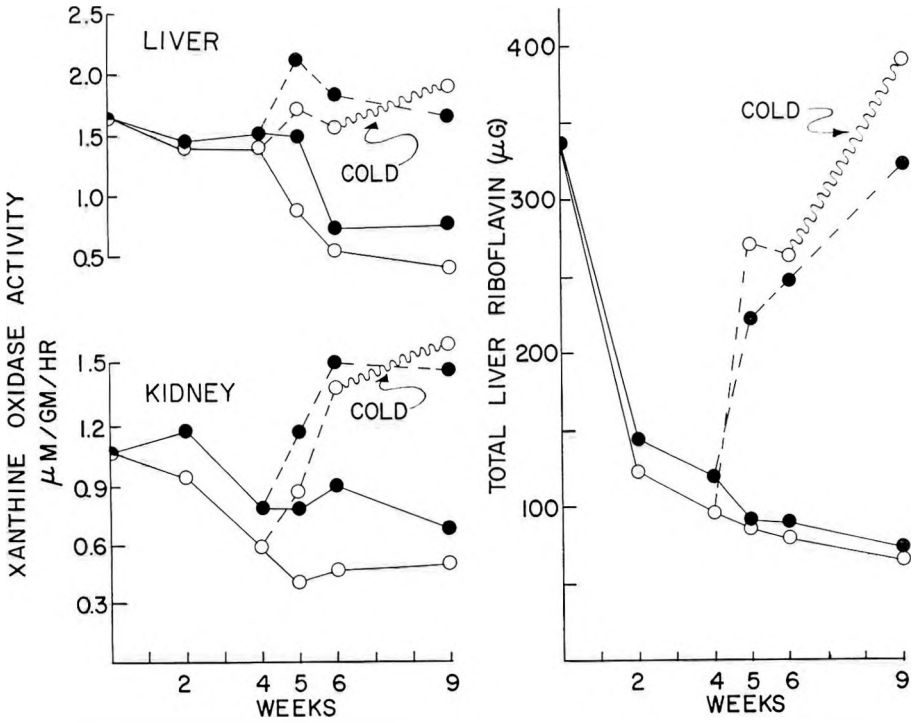

Fig. 4 Xanthine oxidase and riboflavin levels. ○, warm; ●, cold and post-cold; dashed lines, repletion; solid lines, depletion.

resume a slow growth rate and postponed the onset of gross clinical symptoms of the deficiency. This response was similar to that already observed in pantothenic acid-deficient rats (Vaughan and Vaughan, '60). Although these phenomena were reproduced in experiment 2, the additional data obtained have not, in our opinion, provided any definite clues concerning the fundamental reasons for this response. Therefore, a discussion of the results must be, of necessity, predominantly descriptive.

As shown by Bessey et al. ('58), increased energy expenditure induced by cold exposure did not hasten the depletion of liver riboflavin of the deficient rats. As a matter of fact, the *concentration* of liver riboflavin in the deficient cold animals remained about 20% higher than that of the warm-deficient group (Fontaine and Raffy, '42). In terms of liver riboflavin available to the whole animal, this increase was even greater, for the liver weight-to-body weight ratio was higher in the cold animals than in the warm. These cold rats appear thus to conserve some riboflavin (in the liver, at least) while losing weight more rapidly than the warm animals.

Liver xanthine oxidase activity did not decrease until well after liver riboflavin levels had fallen to about one-third of normal, suggesting that this flavoprotein resists dissociation during the initial stages of riboflavin deficiency. The differences between these results and those of Burch et al. ('56), who observed an immediate drop in xanthine oxidase levels, may be related to the different ages of the experimental animals — ours being somewhat older when supplied with the riboflavin-deficient diet. By 6 weeks, however, the levels in our rats had decreased to the same percentages of initial levels as reported by these authors. In the kidney, xanthine oxidase activity appeared to be more labile, but here the deficient post-cold rats maintained a significantly higher activity during the last 4 weeks of the experiment.

Weight losses in the cold rats appeared to consist of fat and water only — protein was not lost. The lability of body fat and water is illustrated in several phases of experiment 2: (1) during the depletion period, as mentioned; (2) during the post-cold period, when fat and water deposition was resumed to a certain extent; and (3) during the sixth to ninth week of cold exposure of the repleted rats, when total stripped weight remained constant, while water deposition slowed, fat was lost, but protein deposition continued unchanged.

At the end of the first 4 weeks, the deficient cold rats were much smaller than the warm rats. Although their post-cold food intake was not greater than that of the deficient warm rats, they would have been able to utilize more for growth. In other words, their food intake-to-body weight ratio was actually greater. However, the elevation of food intake and, consequently, the growth recovery, were not nearly as great as observed in the experiment with pantothenic acid (Vaughan and Vaughan, '60). One possible factor in this difference may be that these rats were in the cold room only 28 days whereas the rats on the pantothenic acid experiment spent 61 days at 5°C before being returned to the 25°C environment.

In conclusion, it would appear that cold exposure during depletion may modify the subsequent course of a deficiency. Whether this is due to a general increase in energy turnover or a specific effect of cold is an intriguing question and, in our opinion, merits further study.

SUMMARY

The post-cold behavior of riboflavin-deficient rats was studied. It was observed that rats depleted at 5°C for 4 weeks resumed slow growth (due to fat and water deposition) upon return to 25°C. Gross symptoms of the deficiency did not appear as soon in the post-cold rats as in the warm rats. Total riboflavin losses from the liver were rapid initially, leveling out at about one-third of normal concentration, but there was little difference between groups. Liver and kidney xanthine oxidase activity did not decrease as rapidly as did riboflavin and the post-cold rats appear to maintain higher levels throughout the experiment.

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Effect of Feeding Animal Tissues on "Meat Anemia" in Mice[1]

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In previous publications it was reported (Adler, '58; Ilan et al., '60; Ilan and Guggenheim, '60) that mice, when fed a diet composed of beef muscle only, developed severe anemia which was mainly macrocytic. Supplementation of the diet with iron, folic acid, vitamin B_{12} or pyridoxine or with a combination of some of these hematopoietic substances neither prevented nor cured the anemia. Incorporation of liver into the meat diet, however, prevented the anemia. Moreover, replacement of one of 4 parts of beef muscle by beef liver cured the anemia completely. Liver of normal mice had a definite but smaller effect, whereas liver of mice rendered anemic by the meat diet resulted in no improvement. It was concluded that beef liver contains a hematopoietic factor needed by mice which is absent from muscle; this hypothetic factor appears to be present in normal mouse liver in a lower concentration than in beef liver and to be absent from the liver of mice that have subsisted on beef muscle.

In a search for the occurrence of this hematopoietic factor, muscle and liver of animals of different species and various beef organs were fed to mice and their anemia-inducing or -preventing potency was studied. Results will also be presented on the effect of feeding different liver preparations on the prevention of meat anemia in mice.

METHODS

Male Swiss mice, three weeks old, were used throughout. They were fed minced raw muscle meat for 6 weeks, if not otherwise stated. After that time hemoglobin was determined by the cyanmethemoglobin method (Crosby et al., '54).

RESULTS

In the first series of experiments the effect of feeding different kinds of muscle was compared (table 1). Skeletal muscle of beef induced a severe anemia, whereas heart muscle prevented it. Mice fed pork or chicken muscle also became anemic, with those fed chicken somewhat less anemic than those subsisting on beef or pork. Skeletal muscle of fish (haddock, hake), in contrast with mammalian and avian skeletal muscle, prevented anemia.

It may be concluded, that the hypothetical hematopoietic factor required by mice is absent from skeletal muscle of beef, pork and chicken and present in fish muscle and heart muscle of beef.

Next, the hematopoietic potency of different beef organs was studied. Liver, kidney, spleen or brain, respectively, were incorporated into minced muscle meat at a level of 25% and fed to mice. The results are shown in table 2. It appears that only liver exerts a strong hematopoietic effect. Kidney and spleen were less effective and brain almost inactive.

In further experiments the effect of replacement of one of 4 parts of beef muscle by liver of different animals was studied (table 3). Liver of the two ruminants, beef and sheep, appeared to be the most potent in preventing meat anemia, followed by rat, pig, human and chicken liver. Doubling the amount of chicken liver in muscle meat resulted, however, in an almost complete prevention of anemia.

Received for publication October 23, 1961.
[1] This study was supported by research grant A-4385 from the National Institutes of Health, Bethesda, Maryland and by the Hematology Research Foundation, Chicago.

TABLE 1

Weight and hemoglobin of mice kept for 6 weeks on diets composed of muscle meat only

Source and kind of muscle	No. of mice		Weight		Hemoglobin	
	Initial	Survivors	Initial[1]	Increase	Initial[1]	Change
			gm	*gm*	*gm/100 ml*	*gm/100 ml*
Skeletal muscle, beef	35	29	12.3 ± 0.40[2]	3.9 ± 0.61	14.7 ± 0.31	− 12.2 ± 0.62
Heart muscle, beef	35	31	13.0 ± 0.29	9.8 ± 0.60	15.2 ± 0.20	− 1.2 ± 0.45
Skeletal muscle, pork	20	16	13.0 ± 0.48	7.2 ± 0.80	14.4 ± 0.37	− 10.2 ± 0.97
Skeletal muscle, chicken	10	6	13.8 ± 0.46	3.2 ± 1.31	14.5 ± 0.49	− 9.3 ± 0.80
Skeletal muscle, haddock	15	10	12.7 ± 0.17	4.5 ± 0.58	14.1 ± 0.54	− 1.2 ± 1.07
Skeletal muscle, hake	15	12	15.3 ± 0.80	4.1 ± 1.22	15.2 ± 0.32	− 2.9 ± 0.80

[1] Figures refer only to survivors.
[2] Figures indicate means ± standard errors.

TABLE 2

Effect of beef organs incorporated into a meat diet on weight increase and prevention of anemia in mice

Beef organ	No. of mice		Weight		Hemoglobin	
	Initial	Survivors	Initial[1]	Increase	Initial[1]	Change
			gm	*gm*	*gm/100 ml*	*gm/100 ml*
Liver	10	7	12.3 ± 0.50[2]	7.1 ± 0.90	15.1 ± 0.48	+ 0.7 ± 0.50
Kidney	10	6	12.4 ± 0.35	8.5 ± 0.79	15.7 ± 0.38	− 2.7 ± 0.79
Spleen	15	11	12.8 ± 0.26	6.5 ± 0.47	15.1 ± 0.47	− 6.2 ± 1.01
Brain	10	7	13.4 ± 0.40	6.3 ± 0.78	15.9 ± 0.31	− 10.1 ± 0.99

[1] Figures refer only to survivors.
[2] Figures indicate means ± standard errors.

The anemia-preventing potency of beef liver was not destroyed by heat. Autoclaving liver for one hour at 15 pounds did not alter its activity. Furthermore, desiccated liver appears to be as active as fresh liver. When 5% of desiccated "mammalian liver" [2] or of desiccated "beef liver," [3] the rough equivalent of 25% of fresh liver, were incorporated into the meat diet, anemia was prevented. Two per cent of desiccated liver was less active and 1% almost inactive.

A significant mortality occurred in all experiments; this, however, did not appear to be related to the specific dietary regimen or to the severity of the resulting anemia.

DISCUSSION

These results are compatible with the hypothesis that "meat anemia" in mice is caused by the deficiency of an hematopoietic factor required by mice and absent from muscle meat. The factor appears to be lacking in muscle meat of pork and chicken and to be present in heart muscle of beef and fish muscle. Liver has the highest anemia-preventing potency, followed by heart muscle, kidney, spleen and brain. Livers of various animals apparently contain the factor in different concentrations. It is highest in beef liver;

[2] Armour Laboratories, Hampden Park, Eastbourne, England.
[3] Obtained from Messrs. Diapharm, Amsterdam, Netherlands.

TABLE 3

Effect of liver of various animals and of various liver preparations on weight increase and prevention of "meat anemia" in mice

Source of liver or of liver preparation	Amount incorporated into meat	No. of mice		Weight		Hemoglobin	
		Initial	Survivors	Initial[1]	Increase	Initial[1]	Change
	%			*gm*	*gm*	*gm/100 ml*	*gm/100 ml*
Beef, raw	25	10	7	12.3 ± 0.50[2]	7.1 ± 0.90	15.1 ± 0.48	+ 0.7 ± 0.50
Sheep	25	10	8	12.0 ± 0.50	7.6 ± 1.90	14.9 ± 0.41	− 1.3 ± 0.65
Rat	25	10	9	11.4 ± 0.42	12.6 ± 0.78	16.0 ± 0.36	− 2.7 ± 0.65
Pig	25	15	10	13.2 ± 0.32	7.1 ± 0.56	15.5 ± 0.44	− 3.3 ± 0.76
Human	25	10	8	12.5 ± 0.51	7.5 ± 0.91	13.9 ± 0.67	− 3.9 ± 0.85
Chicken	25	15	9	13.2 ± 0.26	7.3 ± 0.99	15.7 ± 0.36	− 5.0 ± 1.38
Chicken	50	15	10	12.6 ± 0.34	7.8 ± 1.11	15.3 ± 0.42	− 1.8 ± 0.79
Beef, autoclaved[3]	25	10	7	13.2 ± 0.44	7.2 ± 1.65	14.7 ± 0.47	+ 1.5 ± 0.58
Desiccated liver, Armour[4]	5	15	12	11.5 ± 0.39	10.8 ± 0.95	14.7 ± 0.38	+ 0.6 ± 0.40
Desiccated liver, Diapharm[5]	5	20	15	13.3 ± 0.47	9.7 ± 0.74	15.4 ± 0.28	+ 0.2 ± 0.32
Desiccated liver, Diapharm	2	15	12	13.1 ± 0.37	7.8 ± 0.49	16.5 ± 0.30	− 3.4 ± 0.51
Desiccated liver, Diapharm	1	15	11	14.2 ± 0.40	7.0 ± 0.94	15.7 ± 0.36	− 7.9 ± 0.83

[1] Figures refer only to survivors.
[2] Figures indicate means ± standard errors.
[3] One hour at 15 pounds.
[4] Mammalian liver, obtained from Armour Laboratories, Hampden Park, Eastbourne, England.
[5] Beef liver, obtained from Messrs. Diapharm, Amsterdam, Netherlands.

next follow the liver of sheep, rat, pig, human and chicken. Mouse liver seems to be a poor source as indicated by a curative experiment (Ilan and Guggenheim, '60). The factor is heat-stable and present in desiccated liver at approximately fivefold the amount observed in fresh liver.

It seems, therefore, that various animal tissues, and particularly liver, contain an antianemic factor other than iron, folic acid, vitamin B_{12} or pyridoxine. The occurrence in crude liver extracts of such a factor(s) has been claimed repeatedly (Wintrobe, '56).

The meat diet, even when supplemented with liver appears to be nutritionally inadequate, as observed from the depressed growth and the mortality of mice subsisting on it. The weight of young mice maintained with a stock diet or a semisynthetic full diet increased in 6 weeks by 14.6 and 13.7 gm, respectively, and all survived that observation period (Ilan et al., '60). It may be interesting that the nutritional inadequacy of a meat diet for small rodents was observed 100 years ago (Savory, 1863).

Mice appear to be particularly susceptible to the anemia-producing effect of a meat diet. No anemia occurred in young rats fed the same diet for 12 weeks.[4]

SUMMARY

Young mice maintained with a diet of muscle meat of beef, pork or chicken developed a severe anemia. When the diet consisted of heart muscle of beef or of fish muscle no anemia occurred.

Replacement of one of 4 parts of beef muscle by liver completely prevented the anemia. Kidney and spleen were less effective, and brain was almost ineffective.

Livers of various animals exhibited different anemia-preventing potencies. The most potent were beef and sheep, followed by rat, pig, human and chicken liver. Severe heat treatment or drying of the liver did not destroy its anemia preventing potency.

ACKNOWLEDGMENT

The technical assistance of B. Shapiro and M. Fostick is gratefully acknowledged.

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Comparative Studies of the Growth-Stimulating Properties of Phenothiazine Analogs in the Rat

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The growth-stimulating properties of chemical additives to animal feeds have been observed in domestic animals for antibiotics (Jukes and Williams, '53; Stoksted, '54), and hormones, arsenical and tranquilizers (Ellis et al., '60; Schultze, '60). This stimulation of growth in animals has been observed almost exclusively either with nutritionally inadequate diets or in "diseased" states.

Mathues et al. ('59) reported a significant improvement in body weight gains of vitamin B_6-deficient rats administered a phenothiazine tranquilizer, chlorpromazine hydrochloride.[1] The same dose levels of chlorpromazine that stimulated growth in vitamin B_6-deficient male and female rats depressed growth in animals fed a nutritionally adequate basal diet. Sulman and Winnik ('56) and Rupp and Paschkis ('57) also observed similar growth-depressing effects from relatively high levels of chlorpromazine in male and female rats maintained with nutritionally adequate diets.

Extensive field testing of various tranquilizers for growth enhancement activity in domestic animals appear to have been inconclusive (Andrews et al., '58; Burroughs et al., '59; Hale et al., '59; Hansard et al., '59; Kolari et al., '59; Ralston and Dyer, '59; Jordan and Hanke, '60; Preston et al., '61).

Observations in human subjects have been published showing that weight gains occurred in some patients treated chronically with phenothiazine tranquilizers (Klett and Caffey, '60). These data confirmed previous reports of weight gains accompanying phenothiazine treatments (Bonafede, '57; Planansky, '58; Planansky and Heilizer, '59). Most investigators have implied that there was a correlation between weight gain and increased food consumption in the treated patients. Although it was theorized by these investigators that increased food consumption was related to stimulation of appetite by the drugs, no positive relationship between weight gain and dosage level could be established.

The present paper describes the studies in rats used to assess the potential growth-stimulating properties of analogs of phenothiazine possessing established tranquilizing properties. Data are reported on gains in body weight in weaning and young adult male and female rats and in young adult castrate males. Efficiency of food utilization is reported from studies with young adult male rats. A structure-activity relationship to growth-stimulating properties of phenothiazine analogs is also discussed.

EXPERIMENTAL

Groups (10 rats each) of Long-Evans strain rats from the colony bred and maintained in this laboratory were used in this study. Starting body weight ranges of the experimental rats used were the following: immature males, 50 to 55 gm; immature females, 45 to 50 gm; young adult males, 170 to 190 gm; young adult females, 150 to 170 gm; young adult castrate males (castrated three weeks prior to use), 180 to 200 gm. All animals were housed in individual wire-bottom cages and kept in a controlled environment room (76° ± 2°F, 50% humidity). Synthetic basal diets and tap water were supplied ad libitum. The composition of the synthetic basal diet is detailed in table 1. Body weight gains were measured weekly. Food consumption data were obtained during the last week

Received for publication October 13, 1961.
[1] Thorazine.

TABLE 1
Composition of synthetic basal diet

	%
Casein	20.0
Sucrose	51.5
Cellulose, non-nutritive[1]	4.0
Salts mixture (USP 14)	4.0
Vitamin A and D sucrose mixture[2]	0.5
Vitamin E sucrose mixture[3]	1.0
Choline chloride dextrose mixture[4]	5.0
Multivitamin sucrose mixture[5]	4.0
Cottonseed oil	10.0
Total	100.0

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[2] 10,000 USP units of vitamin A and 1,000 units of vitamin D_2/kg of diet.
[3] 125 IU of *dl*-α-tocopheryl acetate/kg of diet.
[4] 1.20 gm of choline chloride/kg of diet.
[5] Contained in mg/kg of diet: thiamine·HCl, 10; riboflavin, 10; pyridoxine·HCl, 10; Ca-D-pantothenate, 60; niacin, 60; menadione, 200; ascorbic acid, 200; biotin, 1; folic acid, 10; *p*-aminobenzoic acid, 400; inositol, 800; vitamin B_{12} (0.1%), 150.

of the 8-week experimental period in the groups of young adult male rats.

Because earlier phenothiazine studies had shown that maximal growth stimulation occurred within a narrow dose range, with a maximum around 75.0 μg per kg of body weight per day, the constant dosage levels selected for this study were 37.5, 75.0 and 150.0 μg per kg of body weight per day. The compounds were incorporated into the synthetic basal diet and the amounts adjusted weekly for each treatment group to compensate for the increased average body weight of the rats.

The following phenothiazine analogs were used in this study: (a) 10-(3-dimethylamino-2-methylpropyl)-2-trifluoromethyl phenothiazine, hydrochloride, (SK&F 5354-A); (b) 10-(3-dimethylamino-2-methylpropyl)-2-methylthiophenothiazine, hydrochloride, (SK&F 6270-A); (c) 10-[3-(1-methyl-4-piperazinyl)-2-methylpropyl]-2-trifluoromethylphenothiazine, dihydrochloride, (SK&F 5657-A_2); (d) N,N-dimethyl-10-[3-(1-methyl-4-piperazinyl) propyl]-2-phenothiazine sulfonamide, dimethanesulfonate, (SK&F 5883-J_2); (e) 10-[3-(1-[2-(2-hydroxyethoxy)ethyl]-4-piperazinyl) propyl]-2-trifluoromethylphenothiazine, dihydrochloride, (SK&F 7261-A_2); (f) rac-10-(3-dimethylamino-2-methylpropy)-phenothiazine, tartrate, (SK&F 5277-F); and (g) 10-[3-(1-methyl-4-piperazinyl)-propyl]-2-trifluoromethylphenothiazine, dihydrochloride, (SK&F 5019-A_2).

RESULTS

Growth studies. Periodic testing of the synthetic basal diet used in this study has shown it to be equal to a commercial natural diet[2] for optimal growth of the rat. Representative growth curves of body weight gains in young adult male rats are presented in figure 1.

Dose range studies were performed in the young adult male rat using SK&F 5354-A at levels of 37.5, 75.0 and 150.0 μg per kg per day (fig. 2). Significant increases in body weight gain over that of control rats were observed with 75.0 μg per kg per day ($P < 0.01$) and with 150.0 μg per kg per day ($P < 0.05$) at the end of the 8-week experimental period. No significant improvement in growth was observed when the compound was fed at a level of 37.5 μg per kg per day.

A similar dose range in young adult male rats was performed using SK&F 5657-A_2 (fig. 3). At a dietary level of 75.0 μg per kg per day, SK&F 5657-A_2 significantly enhanced ($P < 0.001$) body weight gain over control weight gain. No significant increase of weight gain was observed at either the 37.5 or 150.0 μg per kg per day levels.

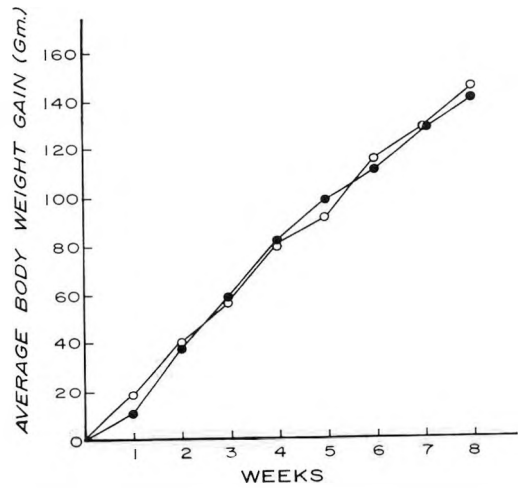


Fig. 1 Comparison of the growth curves of young adult male rats fed synthetic basal and commercial laboratory chow diets; key: ○——○, synthetic diet; ●——●, commercial laboratory chow diet.

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

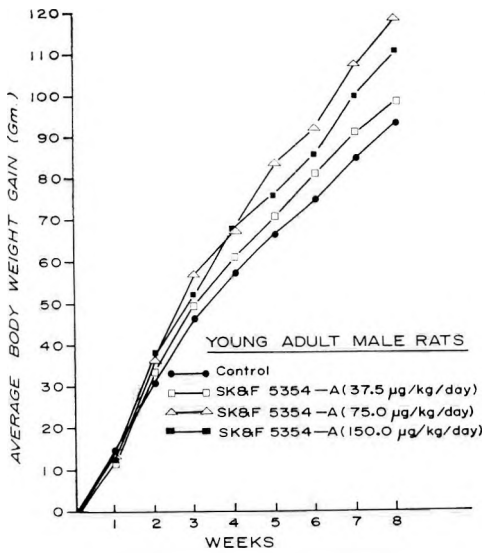


Fig. 2 Dose range studies of the effects of SK&F 5354-A on body weight gain of young adult male rats.

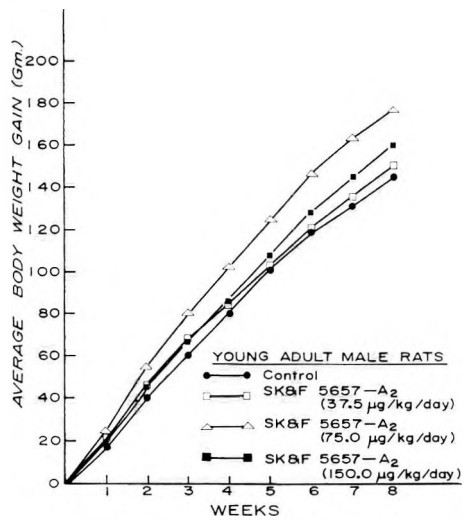


Fig. 3 Dose range studies of the effects of SK&F 5657-A_2 on body weight gain of young adult male rats.

In a subsequent experiment the optimal dose levels of SK&F 5354-A and SK&F 5657-A_2 were compared for growth-enhancing activity in young adult male rats (fig. 4). Statistical analysis of the growth pattern of the rats showed that both compounds significantly increased ($P < 0.05$) weight gain of the treated rats over that of the control rats beginning with the second week of the experiment. These differences became highly significant ($P < 0.01$) at the end of the fifth week and increased in significance ($P < 0.001$) at the end of the 8-week experimental period. No significant difference was observed in body weight gain between the two groups of drug-treated rats.

Optimal dose levels were determined for 5 additional phenothiazine analogs, SK&F 6270-A, 5883-J_2, 7261-A_2, 5277-F, 5019-A_2, and, in each case, this level was observed to be 75.0 µg per kg of body weight per day. Comparative growth studies between SK&F 5354-A and the above 5 compounds at 75.0 µg per kg of body weight per day were carried out on young adult male rats. In addition, a basal control group was included in each study.

Significantly enhanced growth was observed for each of the phenothiazine analogs over that of the basal controls ($P < 0.01$ for SK&F 5354-A, 5883-J_2, 5277-F, 5019-A_2 and $P < 0.05$ for SK&F 6270-A, 7261-A_2). Substitutions on the phenothiazine moiety and the degree of growth enhancement (relative to SK&F 5354-A) are listed in table 2.

In an investigation of the effect of sex of the rat on the growth-stimulating property of the phenothiazines, SK&F 5354-A and SK&F 6270-A were tested in young adult female rats (fig. 5). SK&F 6270-A

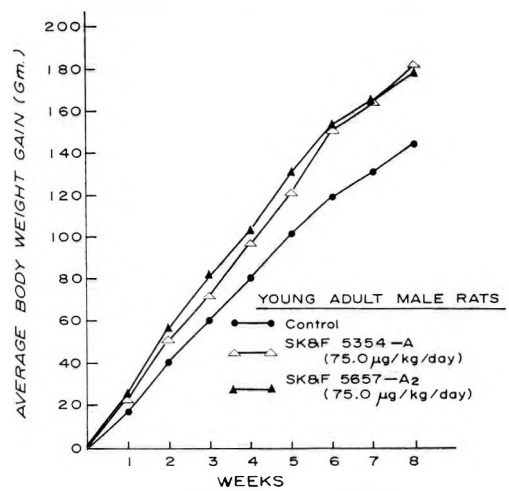
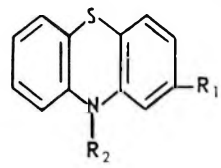


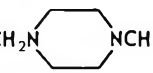
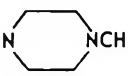
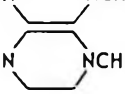
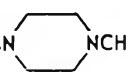
Fig. 4 Comparison of the growth-stimulating effects of SK&F 5354-A and SK&F 5657-A_2 fed at a level of 75.0 µg/kg/day on body weight gain of young adult male rats.

TABLE 2

Structure activity relationship



Phenothiazine Moiety

	R_1	R_2	Growth Stimulation Activity (Relative to SK&F 5354)
SK&F 5354	$-CF_3$	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	
SK&F 5657	$-CF_3$	$-CH_2CH(CH_3)CH_2N$  NCH_3	=
SK&F 5019	$-CF_3$	$-CH_2CH_2CH_2N$  NCH_3	=
SK&F 7261	$-CF_3$	$-CH_2CH_2CH_2N$  $NCH_2CH_2OCH_2CH_2OH$	<
SK&F 5277	$-H$	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	<
SK&F 6270	$-SCH_3$	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	<
SK&F 5883	$-SO_2N(CH_3)_2$	$-CH_2CH_2CH_2N$  NCH_3	<

at 15.0 and 75.0 μg per kg of body weight per day had no apparent effect; SK&F 5354-A significantly depressed ($P < 0.05$) weight gain below that of the control female group.

Because growth enhancement was observed when the phenothiazines were fed to young adult intact male rats but not when fed to young adult female rats, a series of studies were designed to pursue the problem of sex-linked growth responses. Because of these growth differences the study was continued in young adult castrate male rats (fig. 6). Body weight gains of the castrate male rats were not increased by SK&F 5354-A or by SK&F 6270-A, both fed at 75.0 μg per kg per day, over the gains attained by control castrate male rats.

These observations next led to the use of immature male and female rats for growth-enhancement studies. Supplementing the diet of immature male rats with either 75.0 or 150.0 μg of SK&F 5354-A per kg per day did not significantly improve body weight gains when compared with those of the control rats during the 8-week experimental period (fig. 7). However, as the animals matured during growth, increased body weight gain became evident but never became significantly greater than that of the controls. In a parallel experi-

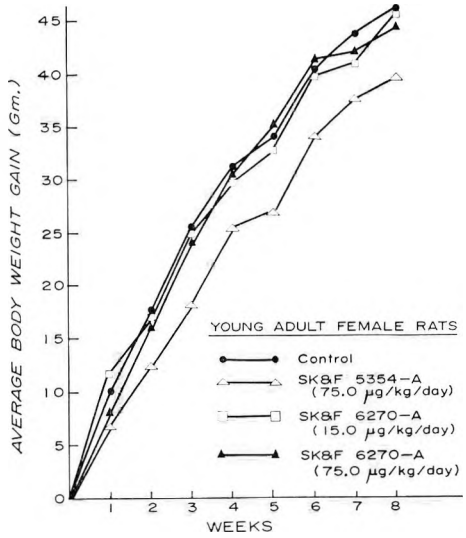


Fig. 5 A study of the growth-stimulating effects of SK&F 5354-A and SK&F 6270-A in young adult female rats.

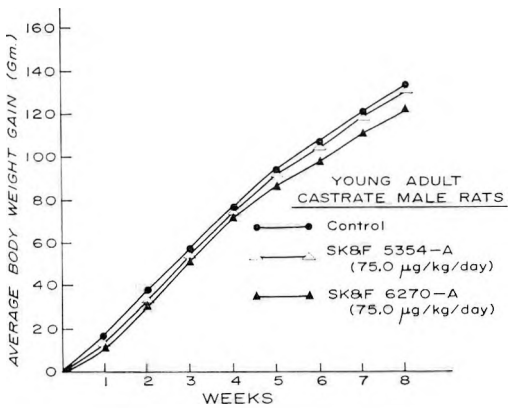


Fig. 6 Growth curves of body weight gains of young adult castrate male rats fed diets supplemented with SK&F 5354-A and SK&F 6270-A.

ment using immature female rats, SK&F 5354-A at similar levels in the diet had no growth-enhancement effect, and contrary to the observations with the immature male rats, little or no increase in growth occurred in the phenothiazine-supplemented rats as they matured (fig. 8).

Efficiency of food utilization. Food consumption was recorded only for young adult male rats in several of the experiments recorded in this report (table 3). From these data and from the body weight gain of the rats, the efficiency of food utilization was calculated. All groups of animals receiving a phenothiazine analog in the diet consumed more food than the control animals. All treated animals were observed to gain more body weight than the control rats during the eighth week of the experiment. Despite the increase in food consumption, all groups of optimally supplemented rats that were observed to gain significantly more weight than the controls

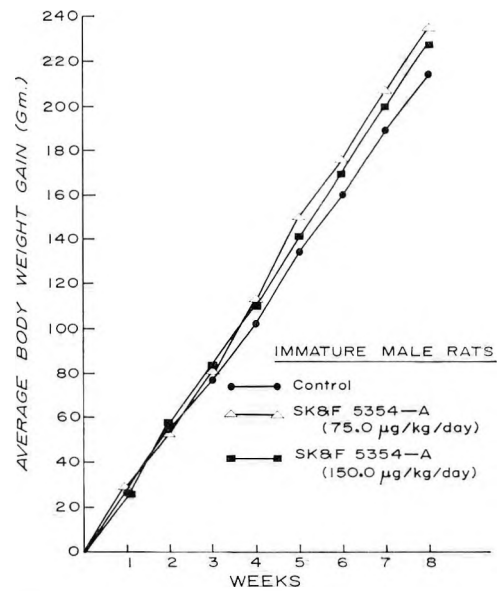


Fig. 7 Studies of the growth-stimulating properties of SK&F 5354-A on body weight gain of immature male rats.

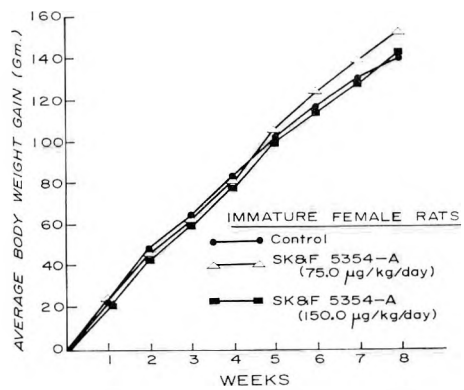


Fig. 8 Studies of the growth-stimulating properties of SK&F 5354-A on body weight gain of immature female rats.

TABLE 3

Comparative studies of the effects of phenothiazine analogs on the efficiency of food utilization in the young adult male rats

Phenothiazine analogs[1]	Amt. dietary drug supplement	Food consumed/ rat/week[2]	Av. body weight gain/ rat/week	Amt. food consumed/ gm weight gain	Efficiency of food utilization[3]
	μg/kg body weight/day	*gm*	*gm*	*gm*	*%*
Control	none	116.2	11.3	10.28	100.0
SK & F 5354-A	37.5	126.5	14.3	8.85	116.2
SK & F 5354-A[4]	75.0	126.7	15.5	8.17	125.8
SK & F 5354-A	150.0	123.6	14.0	8.83	116.4
SK & F 6270-A	75.0	128.2	15.5	8.27	124.3
Control	none	112.4	18.1	6.21	100.0
SK & F 5354-A[4]	75.0	122.9	22.5	5.46	113.8
SK & F 5657-A_2	37.5	119.4	18.8	6.29	98.7
SK & F 5657-A_2	75.0	125.7	22.1	5.69	109.1
SK & F 5657-A_2	150.0	114.1	20.0	5.71	108.7
Control	none	118.0	18.1	6.52	100.0
SK & F 5354-A[4]	75.0	120.2	21.7	5.54	117.6
SK & F 5883-J_2	75.0	125.4	20.9	6.00	108.7
SK & F 7261-A_2	75.0	129.1	20.1	6.42	101.6

[1] Results of three separate studies are detailed.
[2] Food consumption studies determined during 8th week in young adult male rats fed the synthetic diets.
[3] Control groups are arbitrarily assigned a rating of 100.0%.
[4] Reference compound at dietary level producing maximum growth enhancing effects.

were calculated also to have higher efficiency of food utilization than the controls.

DISCUSSION

Early inconsistent reports of growth enhancement with phenothiazine tranquilizers in field trials with domestic animals and reports of obesity in schizophrenic patients receiving tranquilizer therapy led to the initial laboratory evaluation of these phenomena. The laboratory experiments revealed that in order to obtain consistent growth enhancement certain experimental conditions must be considered. Erratic results were observed in early laboratory trials and this led to a strict protocol for experimental procedures for utilizing phenothiazines as growth-enhancement agents. Because phenothiazines are known to be sensitive to factors such as heat, light and oxygen, it was essential that diets be prepared frequently, kept refrigerated until needed, and that all food and glass food cups be changed daily.

It was observed very early in our studies that a narrow dose-response curve was obtained with those phenothiazines tested under the experimental conditions. Because the dietary levels of the phenothiazines are so small compared with those required to show grossly observable pharmacological activity, it would be easy to overdose and completely miss the growth-enhancing activity of these analogs. With the phenothiazines studied, the optimal dose level consistently approximated 75 μg per kg of body weight per day (computed in the 200-gm rat consuming 15 gm of food per day to be 1 ppm. in the food). Dosage levels of the phenothiazines at one-half and twice this level consistently gave less growth stimulation than did the optimal 75 μg per kg per day level.

Appetite stimulation was apparent in all groups of animals receiving the phenothiazine analogs. But, increased body weight gain in the treated animals was not due to increased food intake alone but rather to a combination of increased food intake and greater efficiency of food utilization. Generally the increased food efficiency was greatest in those groups of rats receiving dietary levels of the compounds that exhibited maximal growth-stimulating activity.

Although all of the phenothiazine analogs studied were found to produce growth stimulation, substitutions on the phenothiazine moiety changed the degree of the enhancing activity of the compound (table 2).

The most highly active analogs tested contained a trifluoromethyl substitution at R_1. Substitutions on the R_2 position showed that the methyl radical in the 2-position on the propyl side chain was not necessary for activity and that a methyl piperazinyl group was not significantly different in growth activity for a dimethylamino group. Removal of the trifluoromethyl substitution at R_1, or replacement at this position with a thiomethyl or a sulfonamide group, resulted in some decrease in growth-stimulating activity. Reduction in growth-stimulating properties also occurred with the addition of an ether linkage or lengthening of the side chain at the R_2 position on the phenothiazine moiety.

The data indicate that the effectiveness of the phenothiazines as growth-enhancing agents is sex-linked. No significant enhancement of growth was observed in the studies with the phenothiazines administered to young adult female rats or to immature male or female rats. SK&F 5354-A, at 75 μg per kg of body weight per day actually caused a depression in rate of growth of female rats. Furthermore, it was observed that castration of young adult male rats made the animals insensitive to the growth-enchancing properties of the compounds. The above observations have led to the conclusion that the growth-stimulating effects of the phenothiazines might be dependent on the presence of the male sex hormone in the test animal.

It might be speculated that a relationship exists between the growth-stimulating activity of the phenothiazines and a sex-linked factor in rats. However, on the basis of the experimental evidence presented in this study it is not possible to clearly define a mechanism of action to explain the growth-stimulating properties of these compounds.

SUMMARY

Growth-stimulating properties and efficiency of food utilization of phenothiazine analogs were studied in the rat. Within a narrow dose range it was observed that certain phenothiazine analogs significantly stimulated the growth of young adult male rats. No significant growth stimulation was observed when these compounds were administered to young adult female rats, immature male or female rats or to young adult castrate male rats.

Food consumption data collected during the eighth week of testing indicated that chronic administration of the phenothiazines stimulated appetite and increased the efficiency of food utilization in young adult male rats.

Structure-activity relationships to growth-stimulating properties of the phenothiazines studied were discussed.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of A. Cappelli, H. Koopman and J. Ettore.

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Excretion of D-Lactic Acid by Humans[1]

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In most of the extensive studies in man of lactic acid excretion (Feldmann and Hill, '11; Ryffel, '09–'10; Lewis et al., '25; Liljestrand and Wilson, '25; Johnson and Edwards, '37) no distinction has been made between the optical isomers. In those instances where L-(+)- and D-(−)-lactic[3] were separately estimated, optical rotation was the means of distinguishing the isomers (Craig, '46). This method is insensitive due to the low molar rotation of lactic acid.

D-Lactic acid is poorly utilized in the mammalian organism (Cori and Cori, '29) although it is significantly but weakly glycogenic in the starved rat. Studies in normal and thiamine-deficient rats indicate that D-lactic acid is excreted normally by this species (van Eys et al., '62). In these studies lactic acid was measured by both a specific enzymatic technique and microbiologically, using a mutant of *Lactobacillus casei*, designated 280-16. This mutant was described by Camien and Dunn ('53), and was the subject of extensive studies by these investigators. It responds also to other D-α-hydroxy acids (Camien and Dunn, '53; '54) and to a lesser extent to D-1,2-alkanediols (Camien et al., '56). However, in urine D-lactic acid comprises apparently a major portion of the growth stimulating substances in urine.

METHODS

The D-lactic acid specific dehydrogenase from *Leuconostoc mesenteroides* was prepared as described elsewhere (van Eys et al., '62). Total lactic acid was measured by the method of Barker and Summerson ('41). D-Lactic acid was estimated microbiologically, with *L. casei* 280-16,[4] using the medium patterned after that described by Camien and Dunn ('53). Its composition is given in table 1.

Twenty-four-hour urine collections were assayed where possible; however, for some patients only early morning specimens were available.

RESULTS AND DISCUSSION

D-Lactic acid excretion in normal human urine. The assay figures for total and D-lactic acid obtained in normal human urine are listed in table 2. Great variation between normals occurred as might be expected, since the activity of the individuals was not equalized. However, a given subject remained apparently fairly constant. As an example: Three different subjects gave, on analysis of two separate 24-hour collections, the following values for total lactic acid (in μmoles per 24 hours): 251 and 263; 745 and 941; and 237 and 330.

Lactic acid excretion rises several hundred fold on exercise (table 3). However, the increase is almost exclusively due to the L- form, the D- form showing only a moderate increase due to exercise. The small increment following exercise may be the result of an increased production, but more likely reflects the competition between the two isomers for reabsorption in the kidney (Craig, '46).

Evidence for the occurrence of D-lactic acid. Since the method used for measuring lactic acid is not absolutely specific for D-lactic acid because other D-α-hydroxy-acids show some growth promting activity, it was important to determine that D-lactic acid was being measured. That the D-lactic acid does not arise from racemization during the manipulations of the assay

Received for publication October 16, 1961.
[1] Supported by a grant from the National Science Foundation.
[2] Investigator, Howard Hughes Medical Institute.
[3] From here on the sign of rotation will be omitted. L-(+)-lactic acid is the natural isomer formed through the action of mammalian lactic dehydrogenases.
[4] We are grateful to Dr. Max S. Dunn for supplying us with a culture of this organism.

TABLE 1

Composition of basal media for L. casei 280-16 in lactic acid determination[1]

Vitamin-free casein hydrolysate[2]	10 ml	Xanthine	25 mg
Tryptic digest of casein[3]	50 ml	Sodium chloride	20 mg
Glucose	40.0 gm	$FeSO_4 \cdot H_2O$	20 mg
Sodium acetate	24.0 gm	$MnSO_4 \cdot H_2O$	15 mg
Ammonium chloride	8.0 gm	Nicotinic acid	10 mg
KH_2PO_4	1.0 gm	Pyridoxine·HCl	10 mg
K_2HPO_4	1.0 gm	Calcium pantothenate	5 mg
$MgSO_4 \cdot 7H_2O$	400 mg	Riboflavin	5 mg
L-Cystine	400 mg	Thiamine-chloride-hydrochloride	5 mg
DL-Tryptophan	400 mg	Pyridoxal-hydrochloride	3 mg
Adenine sulfate	25 mg	Pyridoxamine-dihydrochloride	3 mg
Guanine·HCl	25 mg	PABA	1.0 mg
Uracil	25 mg	PGA	0.1 mg
		Biotin	0.01 mg

[1] Amounts given are for 100 ml of double strength medium.
[2] Nutritional Biochemicals Corporation, Cleveland.
[3] 100 gm of casein, suspended in 100 ml of water, was digested at pH 8 to 9 for 48 hours with 100 mg of trypsin and 100 mg of chymotrypsin.

TABLE 2

Urinary output of D(−) and total lactic acid in normal human urine

Subject	24-hour volume	Total lactic acid		D-Lactic acid		D-Lactic acid
	ml	*μmoles/ml*	*μmoles/ 24 hours*	*μmoles/ml*	*μmoles/ 24 hours*	*%*
B.L.	820	0.31	254.2	0.083	68	26.7
S.A.	1300	0.21	273	0.054	70.2	25.7
M.L.	1920	0.32	614.4	0.091	174.7	28.4
J.J.	1510	0.25	377.5	0.051	77	20.4
K.W.	1290	0.71	916	0.036	46.4	5.1
R.N.	1940	0.25	485	0.083	161	33.2
H.A.	1750	0.21	367.5	0.09	157.5	42.9
R.A.	1240	0.29	359.6	0.127	157.5	43.8
R.H.	1900	0.28	532	0.053	100.7	18.9
B.A.	1150	0.33	379.5	0.10	115	30.3
G.R.	2190	0.24	525.6	0.036	78.8	15
T.O.	1840	0.13	239.2	0.025	46	19.2
G.U.	2450	0.19	465.5	0.033	80.9	17.5
W.O.	960	0.56	537.6	0.057	54.7	10.2
T.F.	660	0.44	290.4	0.165	108.9	37.5
B.H.	1330	0.42	558.6	0.059	78.5	14.1
N.I.	1260	0.49	617.4	0.19	239.4	38.8
W.A.	660	0.51	336.6	0.14	92.4	27.5
G.I.	1080	0.36	388.8	0.08	86.4	22.2
O.W.	1620	0.36	583.2	0.04	64.8	11.1
Average[1]	1444	0.34 ± 0.13	455 ± 154	0.08 ± 0.046	102.9 ± 50	24.42 ± 11

[1] Figures are given with their standard deviation.

TABLE 3

Effects of exercise on urinary lactic acid excretion

Subject	Sample	Total lactic acid	D-Lactic acid	D-Lactic acid
		μmoles/ml	*μmoles/ml*	*%*
M.J.	Before exercise	0.49	0.11	22.4
	After exercise[1]	181.2	1.19	0.66
S.A.	Before exercise	0.29	0.061	21.0
	After exercise	86.8	1.39	1.6

[1] A urine sample was collected before each individual ran two times up and down 9 flights of stairs, then another sample was taken 30 minutes following exercise.

is evident from table 3. The extremely small percentage of D-lactic acid excreted in subject M. J. after exercise indicates that the L-lactic acid is not racemized under the conditions used. That lactic acid was measured was indicated by its isolation. A 24-hour sample of human urine was concentrated *in vacuo* at room temperature. The final concentrate, approximately 200 ml, was acidified with sulfuric acid to congo red and continuously extracted for 48 hours with diethyl ether. The residue from the ether extract was distributed between diisopropyl ether/1.0 N sulfuric acid (Camien et al., '59) for 120 transfers. The volume ratio of the two layers was 2/1.

A single theoretical peak was seen in the Barker-Summerson test on the fractions, which coincided with the position expected from the distribution coefficient of lactic acid (Camien et al., '59). Furthermore, the spectrum of the chromophore in the Barker-Summerson test was identical to authentic lactic acid. α-Hydroxybutyric acid gives a different absorption spectrum. On analysis of the counter-current fractions, growth promoting activity was associated with the lactic acid fractions and but little with any other fraction. The appropriate fractions were pooled, extracted into ether and the ether extracts evaporated to dryness. The residue was active as a substrate for both D- and L-lactic acid specific lactic dehydrogenases. The material was insufficient in quantity to determine an accurate molar optical rotation value.

D-Lactic acid in diseased states. Data obtained on a variety of patients is illustrated in table 4. No significant differences existed between the excretion of patients with a number of different diseases. The percentage of D-lactic acid was largely determined by the degree of immobilization of the patient. Thus one completely paralyzed polio patient excreted practically only D-lactic acid.

TABLE 4

Human urinary output of lactic acid in diverse conditions

Subject	Clinical diagnosis	24-hour volume	Total lactic acid		D(−)Lactic acid		D(−)Lactic acid
		ml	*μmoles/ml*	*μmoles/ 24 hours*	*μmoles/ml*	*μmoles/ 24 hours*	*%*
J.H.	Congestive heart failure	1820	0.13	237	0.058	105.6	44.6
V.S.	Cirrhosis	3240	0.23	745	0.045	145.8	19.6
S.R.	Cirrhosis	2280	0.32	730	0.085	193.8	26.6
L.A.	Adrenal cortical hyperplasia	760	0.33	251			
C.W.	Nephrosis	3220	0.29	934	0.10	322	34.5
L.N.	Exogenous obesity	640	0.42	269	0.10	64	23.8
S.F.	Child 7 days post-operative	430	0.31	133	0.01	4.3	3.2
C.A.	Chronic leukemia		0.20		0.01		5.0
K.N.	Hydrocephaly		0.16		0.01		6.3
N.A.	Nephrosis		1.15		1.0		87.0
H.A.	Chronic polio, chronic pyelonephritis		6.21		1.14		18.4
W.H.	Chronic polio		0.38		0.16		42.1
H.U.	Chronic polio[2]		0.42		0.51		—
T.H.	Chronic polio		0.41		0.08		19.5
K.H.	Pseudohypertrophic muscular dystrophy		0.33		0.28		84.8
	Average	1770	0.78	471	0.24	139	32

[1] In the instances where no 24-hour volume is recorded, only early morning specimens were analyzed.
[2] Excluded from the average.

One case of a child with abnormal lactic acid metabolism was examined.[5] This child excreted 110.8 μmoles per ml of total lactic acid and 7.45 μmoles per ml of D-lactic acid. The percentage of D-lactic acid is, however, only 6.7. Thus, although the absolute amount was high, the relative amount was low and the derangement in the child probably was in the metabolism of L-lactic acid. In this child the amount of D-lactic acid, measured enzymatically, gave 2.5 to 3.0 μmoles per ml. This is again an underestimate (van Eys et al., '62) but indicates the large amount of D-lactic acid excreted by this child.

SUMMARY

Normal humans excrete the unnatural isomer of D-lactic acid. When measured microbiologically the values average 0.08 ± 0.046 μmoles per ml and 102.9 ± 50 μmoles per 24 hours. The values are not significantly altered in a variety of diseases and are only moderately raised by exercise.

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[5] This sample of urine was obtained through the courtesy of Dr. A. F. Hartmann, Department of Pediatrics, Washington University, St. Louis.

Fatty Acid Distribution in Tissues from Hens Fed Cottonseed Oil or *Sterculia foetida* Seeds[1]

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Eggs laid by hens fed a ration containing either crude cottonseed oil or *Sterculia foetida* seeds contained higher levels of stearic acid and lower levels of oleic acid than eggs produced by hens fed a normal laying ration (Evans et al., '61). Cottonseed oil and *S. foetida* seeds contain sterculic and malvalic acids, which are characterized by the cyclopropene ring, give the Halphen reaction, and cause pink white discoloration in stored eggs produced by hens fed them (Shenstone and Vickery, '59, '61). Cyclopropene fatty acids or some other constituent in crude cottonseed oil and *S. foetida* seed oil appear in some way to upset the fatty acid metabolism of the hen so that more saturated and less monoenoic fatty acids are deposited in egg yolk from hens fed these acids than in yolks from hens not fed them. Fatty acid metabolism could be upset either in such a way as to cause preferential deposition of saturated fatty acids in the egg or to cause the formation of more saturated fatty acids by the hen. This experiment was conducted to determine which occurs and to study fatty acid metabolism in the laying hen.

METHODS

Three groups of 12 Single Comb White Leghorn hens per group were kept in individual laying cages and fed a basal ration with the following percentage composition: ground corn, 34.5; ground oats, 20.0; wheat bran, 15.0; flour middlings, 10.0; dehydrated alfalfa (15% protein), 3.0; meat scrap (50% protein), 3.0; dried milk, 2.0; menhaden fish meal (60% protein), 2.5; soybean oil meal (44% protein), 2.5; ground oyster shell flour, 5.0; steamed bone meal, 1.5; salt, 0.6; and fish oil (3,000 units of vitamin A and 400 ICU of vitamin D_3), 0.4. This ration contained 4.1% of ether extractable material. One group was fed the basal ration only. The second group was fed the basal ration plus 2.5% of crude cottonseed oil[3] added at the expense of an equal weight of basal ration. The third group was fed the basal ration plus one pound of ground *S. foetida* seeds[4] in 150 pounds of complete ration. The *S. foetida* seeds added 0.16% of oil to the ration.

The hens were fed these rations for 6 weeks, at the end of which time 6 hens from each group were killed, and blood, liver, heart, ovary with the eggs contained therein, and depot fat samples were obtained. Blood was collected in citrate, and the plasma was separated from the cells by centrifugation. Hearts from the hens in each group were composited. Other tissues were analyzed on an individual hen basis. Blood plasma was frozen at $-60°C$ until analyses were performed. Liver and ovary samples were individually homogenized in a Waring Blendor, shell frozen, and lyophilized, and the dried samples were stored at $-60°C$ until analyzed. Hearts were homogenized by groups and tested in the same way. Depot fat was stored at $-60°C$ and then ground in a meat chopper while still frozen.

Dried livers, hearts, and ovaries were extracted with chloroform-methanol (1:1). The extracts were saponified overnight at

Received for publication October 2, 1961.

[1] Journal article no. 2876 from the Michigan Agricultural Experiment Station. Supported in part by a grant from the National Cottonseed Products Association.

[2] Participant in the National Science Foundation Undergraduate Research Participation Program of the Biochemistry Department during the summer of 1961.

[3] Crude cottonseed oil was kindly furnished by Dr. Phillip D. Aines of Buckeye Cotton Oil Division, Cincinnati, Ohio.

[4] *Sterculia foetida* seeds were obtained from Dr. Hans R. Schmidt, S. B. Penick and Company, New York. These seeds came originally from India.

room temperature with alcoholic potassium hydroxide, the soaps were acidified with dilute sulfuric acid, and the fatty acids were extracted with peroxide-free diethyl ether, as described by Evans et al. ('61). The fatty acids were methylated with diazomethane. Depot fat was mixed with 1.5 times its weight of anhydrous sodium sulfate to remove water and then extracted with chloroform-methanol (1 : 1). The extracts were treated in the same way as the other tissue extracts.

Blood plasma fatty acid methyl esters were prepared by refluxing for one hour with 1% sulfuric acid in methanol using the transmethylation procedure of Michaels ('58).

Fatty acid methyl esters were separated on a diethylene glycol succinate (DEGS) column in an Aerograph Model A-90-C gas-liquid chromatographic instrument operated at 200°C with a helium flow rate of 100 ml per minute. The Aerograph was equipped with a Weston 1-mv single point recorder.

RESULTS

Fatty acid methyl esters from eggs produced by hens fed the basal ration and from hens fed the basal ration plus 2.5% of crude cottonseed oil were prepared and analyzed by gas-liquid chromatography. The gas liquid chromatograms obtained are compared in figure 1, which illustrates how the feeding of crude cottonseed oil decreased the size of the palmitoleic and oleic acid peaks and increased the size of the stearic acid peak.

The fatty acid distributions in the rations fed each group are shown in table 1. Values for the basal ration were determined directly, but values for the other rations were calculated from those for the basal ration and for crude cottonseed oil and *S. foetida* seed oil. None of the rations supplied significant amounts of stearic acid. Cottonseed and *S. foetida* seed oils contained very little stearic acid, and the oleic acid content was just about the same as in the basal diet fat. The *S. foetida* seed supplemented ration contained 1.3% of sterculic acid in the mixed fatty acids, the exact level of malvalic acid in the crude cottonseed oil is not known, because malvalic acid is included with linoleic acid in the gas-liquid chromatographic separation procedure used. According to data of Shenstone and Vickery ('61) the crude oil from the cot-

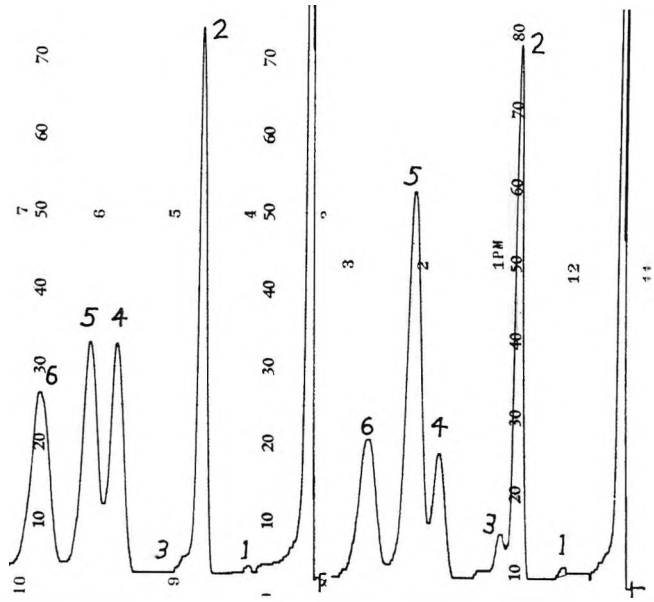

Fig. 1 Gas-liquid chromatograms of methyl esters of fatty acids from yolks of eggs produced by hens fed the basal ration (right) and the basal ration plus 2.5% of crude cottonseed oil (left). Peaks are as follows: 1, myristate; 2, palmitate; 3, palmitoleate; 4, stearate; 5, oleate; and 6, linoleate.

tonseed oil ration should contain about 0.42% of malvalic acid and 0.15% of sterculic acid and the oil from the sterculic ration about 1.88% of total cyclopropene fatty acids. Kemmer,[5] however, estimates the total cyclopropene acids to make up about 1% of crude cottonseed oil according to the Halphen reaction. The total ration oil would contain about 0.38% of cyclopropene acids.

TABLE 1

Fatty acid distribution in oils extracted from rations

	Ration fed		
	Basal ration	Cottonseed oil ration	*S. foetida* seed ration
	%	%	%
Myristic	1.7	1.5	1.6
Palmitic	16.6	20.9	17.0
Palmitoleic	2.7	2.1	2.6
Stearic	3.2	2.8	3.2
Oleic	27.4	23.4	26.7
Linoleic	45.1	47.3	44.1
Linolenic	2.2	1.4	2.1
Sterculic	0.0	0.0	1.3
Eicosenoic	1.2	0.7	1.2
Unknown	0.0	0.0	0.4

Blood plasma lipides from hens fed crude cottonseed oil or *S. foetida* seeds contained more stearic acid and less oleic acid than those from hens fed the basal ration only (table 2). *S. foetida* seeds caused a greater increase in stearic acid and decrease in oleic acid than crude cottonseed oil.

Feeding crude cottonseed oil or *S. foetida* seeds to laying hens increased the stearic acid and decreased the oleic acid distribution in their liver lipides (table 3).

Although the differences were not large, and not statistically significant, stearic acid increased and oleic acid decreased in heart lipides of hens fed crude cottonseed oil or *S. foetida* seeds (table 4).

Most of the hens from this study were in good production, and the ovaries were filled with egg yolks in various stages of development. Some even contained fully developed eggs nearly ready to be laid. One would therefore, expect that the fatty acid distribution in the ovaries would be similar to that in eggs. Although the fatty acid distribution was not determined in

[5] Personal communication.

TABLE 2

Fatty acid distribution in blood plasma lipides

	Ration fed		
	Basal	Cottonseed oil	*S. foetida* seeds
	%	%	%
Myristic	0.1(0.0–0.5)[1]	0.2(0.0–0.5)[1]	0.2(0.0–0.4)[1]
Palmitic	31.3(30.0–33.2)	33.2(30.0–35.9)	32.2(28.4–35.5)
Palmitoleic	2.9(2.5–3.8)	2.3(1.1–4.2)	1.7(1.1–2.8)
Stearic	17.5(15.1–20.7)	22.0(20.8–23.7)	29.0(22.0–38.2)
Oleic	34.5(30.6–39.3)	24.3(22.8–27.4)	20.4(14.0–25.1)
Linoleic	13.7(11.7–16.6)	18.1(17.0–20.9)	16.6(13.8–19.0)

[1] Range of values.

TABLE 3

Fatty acid distribution in hen liver lipides

	Ration fed		
	Basal	Cottonseed oil	*S. foetida* seeds
	%	%	%
Myristic	0.5(0.4–0.6)[1]	0.6(0.4–0.7)[1]	0.5(0.3–0.6)[1]
Palmitic	23.4(22.8–24.1)	26.5(25.7–27.3)	24.9(22.7–25.8)
Palmitoleic	3.1(2.5–3.8)	2.3(1.9–2.7)	2.2(1.7–2.6)
Stearic	14.0(11.2–17.1)	22.2(19.6–26.7)	22.4(17.1–25.8)
Oleic	36.2(31.4–41.1)	27.7(25.7–29.1)	25.2(24.0–27.7)
Linoleic	22.9(19.5–25.4)	20.8(13.7–24.5)	24.9(20.0–27.4)

[1] Range of values.

eggs laid by these hens, the fatty acid distributions in the ovaries (table 5) agree well with those in the eggs produced by hens fed similar diets in a previous experiment (Evans et al., '61). Crude cottonseed oil or *S. foetida* seeds in the ration increased the saturated and dienoic fatty acid contents of the ovaries at the expense of the monoenoic.

Crude cottonseed oil or *S. foetida* seed feeding had little effect on the fatty acid distribution in hen depot fats (table 6). Fats from hens fed crude cottonseed oil or *S. foetida* seeds contain only slightly more stearic acid and slightly less oleic acid than fat from hens fed the basal ration. Depot fat of hens fed *S. foetida* contained between 7.5 and 13.0% of stearic acid and 29.7 and 38.0% of oleic acid.

No values are presented in the tables for linolenic acid or for any fatty acids of longer chain length than 18-carbon atoms, because even though in some instances small amounts of linolenic and arachidonic acids were present, the amounts were too small to give an accurate estimation of the amount with the procedure used. These fatty acid methyl esters emerged from the gas-liquid chromatographic column after methyl linoleate, and the peaks were so broad that unless a considerable amount was present they could not be satisfactorily determined. Although attempts were made to determine methyl linolenate and methyl arachidonate in each tissue studied, the peaks were in all instances too small to obtain reliable results.

TABLE 4

Fatty acid distribution in hen heart lipides

	Ration fed		
	Basal	Cottonseed oil	*S. foetida* seed
	%	%	%
Myristic	0.8	0.8	0.8
Palmitic	19.7	21.4	20.6
Palmitoleic	5.7	3.2	2.8
Stearic	9.3	12.5	13.2
Oleic	32.3	30.7	30.5
Linoleic	32.3	31.6	32.0

DISCUSSION

The basal diet fatty acids contained only 3.2% of stearic acid and 27.4% of oleic acid (table 1), but eggs laid by hens

TABLE 5

Fatty acid distribution in hen ovaries and contents

	Ration fed		
	Basal	Cottonseed oil	*S. foetida* seeds
	%	%	%
Myristic	0.5(0.4–0.7)[1]	0.5(0.5–0.6)[1]	0.6(0.5–0.8)[1]
Palmitic	25.7(24.4–26.5)	28.5(27.3–29.4)	28.5(26.5–30.4)
Palmitoleic	3.6(2.5–4.2)	1.8(1.5–2.0)	1.8(1.5–2.0)
Stearic	10.4(9.2–11.5)	21.4(19.4–23.8)	23.9(21.4–28.0)
Oleic	42.4(40.5–44.6)	26.6(25.6–28.2)	23.0(19.3–25.0)
Linoleic	17.1(15.0–18.6)	21.2(17.2–24.1)	22.2(20.4–23.6)

[1] Range of values.

TABLE 6

Fatty acid distribution in hen depot fats

	Ration fed		
	Basal	Cottonseed oil	*S. foetida* seeds
	%	%	%
Myristic	1.1(1.0–1.2)[1]	1.1(1.0–1.2)[1]	1.1(0.9–1.2)[1]
Palmitic	20.6(19.6–21.4)	23.4(21.6–26.3)	21.0(17.7–24.6)
Palmitoleic	4.0(3.3–4.8)	3.8(2.6–4.8)	3.4(3.1–3.8)
Stearic	6.2(5.6–7.2)	7.9(7.0–8.7)	9.6(7.5–13.0)
Oleic	36.5(34.2–39.5)	34.2(32.2–36.8)	34.9(29.7–38.0)
Linoleic	31.5(29.7–33.0)	29.6(25.3–33.4)	30.0(27.5–31.3)

[1] Range of values.

fed this diet contained 10.4% of stearic acid and 38.1% of oleic acid in the fatty acid fraction (Evans et al., '61). But the ration fatty acids contained 45.1% of linoleic acid and the eggs but 17.5%. Apparently linoleic acid was either partially hydrogenated by the hen before it was deposited in the egg yolk, or most of the egg fatty acids were synthesized from dietary carbohydrates and mostly stearic acid was produced.

The liver is believed to play an important role in fat metabolism, and newly absorbed fat is probably worked over in the liver to alter the nature of its fatty acid pattern so that it will conform to the type usually stored by a particular species (Deuel, '55). One would suspect that conversion of linoleic acid to stearic and oleic acids, if it occurs, occurs in the liver. The liver is also the principal site of conversion of carbohydrate to lipide (Ranney and Chaikoff, '51).

Plasma fatty acids contained more stearic and oleic acids and less linoleic acid (table 2) than dietary lipides (table 1), and liver lipides contained intermediate amounts (table 3). Dietary fatty acids contained 16.6% of palmitic acid and the plasma fatty acids contained 31.3%, but plasma did not contain less palmitoleic acid than dietary fats. Synthesis of palmitic acid from carbohydrates or partial metabolism of linoleic acid to give palmitic acid by loss of a two-carbon unit and saturation is thus indicated.

Cyclopropene fatty acids may be the active agents in crude cottonseed oil and *S. foetida* seeds (Evans et al., '61). The data presented indicate that the cyclopropene fatty acids or some other constituent of *S. foetida* seeds or crude cottonseed oil in some way interfere with the fatty acid metabolism of the hen to cause production of a larger percentage of saturated fatty acids. There are at least two possible modes of action of the active agent. Linoleic acid may be normally hydrogenated to oleic acid and stearic acid, and cyclopropene fatty acids may interfere so that partial hydrogenation does not occur, but most of the linoleic acid is completely hydrogenated to stearic acid. Cyclopropene fatty acids may interfere in the mechanism of conversion of carbohydrate to fatty acid. The basal diet probably did not supply sufficient lipide to meet the hens' requirements for egg production and other body needs. This would necessitate conversion of carbohydrate to lipide. Stearic and palmitic acids were probably first synthesized, and they were normally converted to the corresponding monoethenoid fatty acids (Schoenheimer and Rittenberg, '36). Desaturation of stearic and palmitic acids was, however, blocked when crude cottonseed oil or *S. foetida* seeds were fed. Feigenbaum and Fisher ('59) observed more saturated fatty acids but less linoleic acid in eggs from hens fed cottonseed oil than in those from hens fed a fat-free diet. When crude cottonseed oil or *S. foetida* seeds were fed, the stearic acid content of liver, blood plasma, and ovary lipides increased, but that of depot fat lipides did not even though Tuerkischer and Wertheimer ('42) showed that fat synthesis may occur in adipose tissue.

Ovaries of hens fed the basal ration contained a lower proportion of stearic acid and a higher proportion of oleic acid than either plasma or liver. Thus there appears to be some selectivity in the fatty acids deposited in the egg yolk. Most of the yolk lipides are triglycerides (Evans et al., '61) deposited in the lipovitellenin complex of the yolk (Evans and Bandemer, '61). Lipovitellenin is probably synthesized in the liver and transported as such in the blood to the ovary (McIndoe, '59). If the composition of lipovitellenin in plasma is the same as lipovitellenin in the egg, either the rest of the plasma lipides contained higher levels of stearic acid and lower levels of linoleic acid than lipovitellenin, or some of the lipovitellenin had already been removed from the plasma, and the plasma fatty acids therefore contained less myristic and linoleic acids and more stearic and palmitic acids than the ovary fatty acids or the lipovitellenin.

Much of the depot fat was obtained from around the intestinal tract, but some was taken from other places. The fatty acid distribution in depot fat (table 6) more nearly resembles that of the dietary fat than that of the blood plasma, but for the most part the distribution in depot fat was

between that in plasma and diet. The data, therefore, indicate that much of the depot fat may have been deposited before it was altered in the liver. Probably most of the depot fats were deposited before the hens were put on these experimental rations, so that feeding crude cottonseed oil or *S. foetida* seeds did not have much influence on depot fatty acid composition.

Heart lipides had a characteristic fatty acid pattern that did not appear to be related to either dietary or plasma fatty acid distribution and which was only slightly altered by the presence of cottonseed oil or *S. foetida* seeds in the ration (table 4). The increased stearic acid content of heart fatty acids may be of importance.

The increased stearic acid and decreased oleic acid distribution in liver and blood plasma fatty acids demonstrate that crude cottonseed oil and *S. foetida* seeds interfere with the fatty acid metabolism of the laying hen rather than causing preferential deposition of saturated fatty acids in the egg. The latter would have resulted in a decrease rather than an increase of saturated fatty acids in the blood, and there would have been no change in the liver.

Cottonseed oil also interferes with fatty acid metabolism of other animal species than the hen. Ellis et al. ('31) observed that 4% of cottonseed oil in the ration of pigs increased stearic acid and decreased oleic acid in the back fat when compared with pigs fed a basal ration or a ration containing 4% of peanut, soybean, or corn oils.

SUMMARY

Hens fed either crude cottonseed oil or *Sterculia foetida* seeds in their diets had higher concentrations of stearic acid and lower concentrations of oleic acid in the fatty acids of their livers, blood plasma, and ovaries than hens fed a basal diet. Only slight changes occurred in the fatty acid distribution of heart lipides or depot fat of these hens.

Cottonseed oil and *S. foetida* seed oil interfere with fatty acid metabolism in the hen to produce more saturated fatty acids and less monoenoic fatty acids than produced by hens not fed these oils.

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