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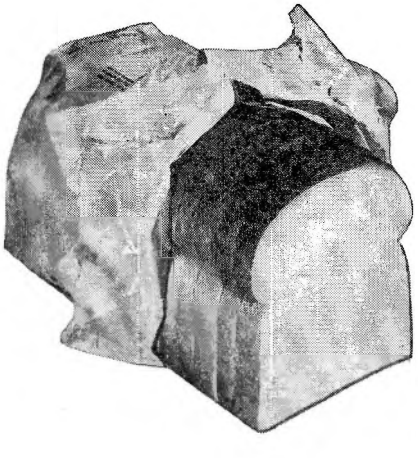

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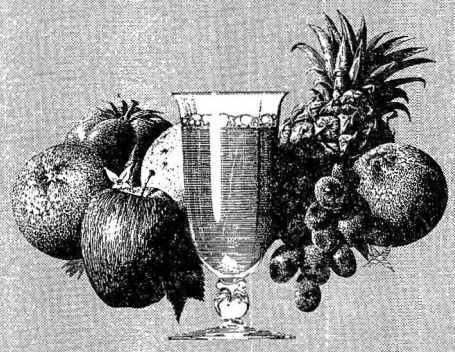
WHY SHOULD THE VITAMIN C CONTENT OF FRUIT AND VEGETABLE JUICES BE STANDARDIZED?



Because nutrition reports disclose that —

- **Vitamin C deficiencies exist throughout the country***
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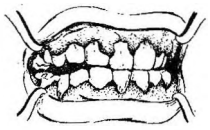
Proof of deficiencies among adults and children. You should know these facts!* Diet surveys from Maine and Vermont, New York, Illinois, New Mexico, Oregon, West Virginia, and Texas show a definite pattern of inadequate vitamin C intake. These studies covered grade school and high school children, college men and women.



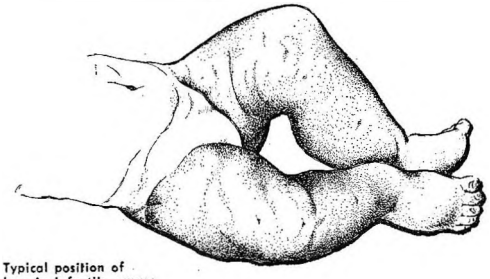
Spongy, swollen gums symptomatic of scurvy.

From these studies* of dietary habits and physical conditions there is considerable proof that many of the children and adults covered by survey have actual, physical symptoms of vitamin C deficiency.

Infantile scurvy. Scurvy* occurs, even today, among infants who are artificially fed. Fortunately the disease may be cured by the administration of vitamin C. How much more sensible it is to prevent it by supplying these bottle-fed infants with the daily allowance of 30 milligrams of vitamin C as recommended by the Food and Nutrition Board of the National Research Council for children under one year of age.

Symptoms of infantile scurvy are fretfulness and fear of being touched.

These bottle-fed infants, unless receiving a pediatric prescription containing vitamin C, are largely dependent on supplemental ascorbic acid which they receive from fruit or vegetable juices. Unless fortified with vitamin C many of the processed juices may not supply these babies w adequate quantities of this essential element.



Typical position of legs in infantile scurvy.

What can be done? Because of the increasing e dence that American diets are deficient in vitamin leading nutrition experts believe that processed fruit a vegetable juices, on which the public is so reliant for supply of this necessary vitamin, should contain t "Vital Ingredient."

The Vital Ingredient of any processed fruit or vegetal juice is a *dependable* and *adequate* supply of vitamin You can give your juice this ingredient by *standardizi its vitamin C content.* The processing is simple and t new low cost of ascorbic acid makes juice standardi tion more attractive than ever.

With ascorbic acid available *by the tons* at extrem modest cost fruit and vegetable juices can have a star ardized vitamin C content for the better protection the nation's health and well-being.

*Statements are based on documented reports. References on requ

Illustrations after photographs in The Vitamins in Medicine by Bick and Prescott, Third Edition, published by Grune & Stratton, Inc., New Yo

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ASCORBIC ACID REQUIREMENT OF THE GUINEA PIG USING GROWTH AND TISSUE ASCORBIC ACID CONCENTRATIONS AS CRITERIA [1]

MACIE COLLINS AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication May 31, 1957)

In estimating the ascorbic acid requirement of the guinea pig less consistent values were obtained when growth was used as the criterion than when estimates were made from the rate of development of specific tissues. Waugh and King ('32) found that 0.5 mg of ascorbic acid per animal per day was adequate for good growth and protection against scurvy. A similar observation was made by Dawborn ('45) who recommended 0.4 mg per day as the requirement. Coward and Kassner ('36) obtained maximum growth with 1.0 mg per day, and Dunker et al. ('42) with 0.6 mg per day.

According to Crampton et al. ('44) scorbutigenic diets in previous studies were nutritionally inadequate. Therefore, attempts to estimate the requirements based on growth as the criterion were complicated by other deficiencies. Crampton found the requirement to be 2.0 mg per day using a natural diet, which when supplemented with ascorbic acid gave optimal growth.

Now that a purified diet, which is nutritionally adequate, has been developed (Reid and Briggs, '53; Heinecke et al., '55), it is possible to study dietary requirements uncomplicated by

[1] Published with approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. The crystalline vitamins were kindly provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

other deficiencies. In view of the variation in the values for the requirement based on growth, this work was undertaken to reinvestigate the requirement of the guinea pig for ascorbic acid.

EXPERIMENTAL

Guinea pigs were housed in screen-bottom cages suspended over wood shavings which were changed daily. Because of the susceptibility of the guinea pig to infection, sanitation was emphasized. Drinking bottles were washed daily and filled with distilled water. Clean food cups were provided regularly.

The purified diet which was fed ad libitum consisted of: casein, 30; sucrose, 40; corn oil, 7.4; salts 4,[2] 4; magnesium oxide, 0.5; potassium acetate, 2.5; choline chloride, 0.35; and roughage,[3] 15. Water-soluble vitamins were added in the following quantities per kilogram of the diet: inositol, 2 gm; niacin, 0.2 gm; para-aminobenzoic acid, 0.1 gm; calcium pantothenate, 0.08 gm; riboflavin, 0.03 gm; thiamine, 0.02 gm; pyridoxine, 0.02 gm; folic acid, 0.01 gm; biotin, 1.0 mg; and cyanocobalamin, 0.04 mg. Each animal received a fat-soluble supplement once a week containing vitamin A, 1,060 I. U.; vitamin D, 10.6 I. U.; vitamin E (α-tocopherol), 7.2 mg and menadione, 0.12 mg.

Young adult guinea pigs were allowed 7 to 14 days for adaptation to the purified diet; one- to two-day old guinea pigs were allowed 21 days.

The ascorbic acid solution (0.1 mg per ml) was prepared daily in 20% sucrose solution. Each guinea pig received the supplement daily. The animals were weighed twice a week and the amount given was based on the body weight recorded at the previous weighing.

Experiment I. Guinea pigs weighing from 350 to 450 gm were divided into groups of 6. After 16 days depletion each animal was given one of the following levels of ascorbic acid: 2.0, 1.0, 0.5, 0.3, 0.25, and 0.1 mg per 100 gm body weight per

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

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

day for 26 days. To determine the effect of depletion on the rate of gain of animals of this age, two groups of 6 animals each were given supplements of 0.3 and 0.25 mg per 100 gm body weight from the beginning of the experiment.

Experiment II. Young guinea pigs weighing from 150 to 200 gm were divided into groups of 10 after 11 days depletion (preceded by 21 days for adaptation) and were maintained for 91 days on one of the following levels of ascorbic acid: 1.0, 0.5, 0.4, 0.3 and 0.2 mg per 100 gm of body weight per day. Also three groups of 6 guinea pigs weighing from 150 to 200 gm were supplemented from the beginning of the experiment with 0.4, 0.3 and 0.2 mg per 100 gm body weight.

Experiment III. The same depletion procedure as that of experiment 2 was used. The levels of ascorbic acid administered were 0.7, 0.5, 0.4, 0.3 and 0.2 mg per 100 gm per day. After 49 days, the animals were anesthetized;[4] a blood sample was removed by heart puncture 20 to 24 hours after supplementation and the liver, adrenals, and spleen were rapidly removed and weighed for analysis. Tissues were prepared for analysis by homogenizing weighed samples in a Potter-Elvehjem homogenizer in 6% trichloroacetic acid. Tissues and whole blood were analyzed for total ascorbic acid by the method of Roe and co-workers ('43) using acid-washed norite as the oxidant.

RESULTS

During the first week of the depletion period, the animals continued to gain weight at an average of from 4 to 6 gm per day, depending upon the age of the animal. By the 14th day, almost all of the older animals were losing weight rapidly and were beginning to show symptoms of scurvy. Post mortem examinations revealed enlarged and hemorrhagic adrenals, hemorrhages in the hind and fore joints, in the intestine and intercostal muscles. Younger animals were losing weight consistently by the 9th day of the depletion period.

[4] Nembutal, Abbott, was used, 5 mg per 100 gm of body weight, by intraperitoneal injection.

In the depletion experiments, since animals that died were generally well below the average weight of the group, the average gain was calculated using only the weight gains of the surviving animals for the previous week. This procedure excluded the abnormally high weight gains that would have otherwise resulted. The final averages used in evaluating the

TABLE 1

Growth and survival on graded levels of ascorbic acid

ASCORBIC ACID	NO. OF ANIMALS	SURVIVORS	AV. GAIN [2]
mg/100 gm			*gm/day*
	With depletion [1]		
2.0	6	6	4.6 ± 2.1 [3]
1.0	6	6	5.8 ± 0.2
	7	7	5.0 ± 1.9
0.5	6	6	5.5 ± 0.2
	7	7	6.1 ± 0.9
0.4	6	6	3.6 ± 1.0
0.3	6	6	3.4 ± 0.8
0.25	7	3	1.4 ± 1.6
0.1	7	2	—3.9 ± 1.4
	Without depletion [4]		
0.3	6	6	3.4 ± 0.9
0.25	6	4	1.5 ± 1.3

[1] Animals were supplemented after 16 days of depletion.

[2] The average gain includes only the weight gains of the surviving animals for the previous week. In the depletion experiment the average gain includes values from the 28th day through the 42nd day. With the non-depleted animals, the average gain includes the entire 42-day period.

[3] Standard error.

[4] Animals were supplemented from the beginning of the experiment.

adequacy of the level of supplementation include only those gains from the 28th day on, as it is only after 28 days that the animals gain weight at a constant rate.

The data obtained in experiment 1 using depleted and non-depleted young adult guinea pigs are given in table 1. After depletion the 2.0, 1.0 and 0.5 mg levels supported a normal weight gain and survival rate; therefore all three levels were adequate for maximum growth. The rate of gain was reduced

as the level of ascorbic acid was decreased to 0.25 mg and the animals receiving only 0.1 mg lost weight. The animals on the 0.3 and 0.4 mg levels grew at an intermediate rate. Only the animals on the two lower levels had sore joints at the conclusion of the experiment.

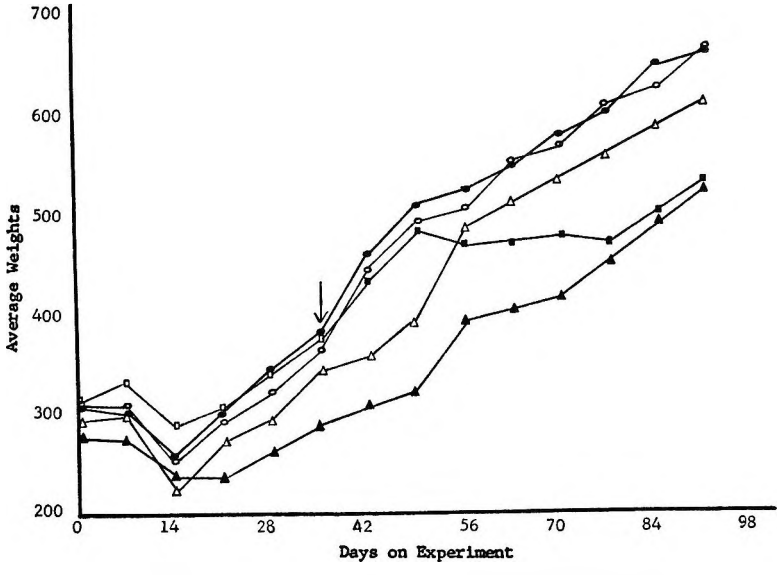

Fig. 1 Growth of guinea pigs supplemented with graded levels of vitamin C after depletion. The amounts of ascorbic acid added per 100 gm of body weight were: □ = 1.0; 0.15 mg
● = 0.5 mg ○ = 0.4 mg △ = 0.3 mg ▲ = 0.2 mg

When the average weight gains of animals after depletion are compared with the average weight gains of animals that had not been depleted, it is seen that depletion does not effect the rate of growth. The growth rates of the depleted and the non-depleted groups on both the 0.25 and 0.3 mg levels (table 1) are comparable.

Growth curves of the 5 depleted groups of experiment 2 are presented in figure 1. Since the curve for the group receiving the 1.0 mg level of ascorbic acid closely followed that of the group receiving the 0.5 mg level, 0.15 mg was substituted for

the 1.0 mg level on the 35th day of the experiment and was continued for the duration of the experiment. The curve for the group receiving 0.15 mg gradually leveled off until the 77th day when the animals began to grow at about the same rate at those receiving 0.2 mg per 100 gm. The group receiving the 0.3 mg level grew much more rapidly than that receiving the 0.2 mg level. The growth rates of the groups receiving the 0.4 and 0.5 mg levels were about the same; however, there is evidence in table 2 that the 0.5 mg level was

TABLE 2

Growth and survival of young animals on graded levels of ascorbic acid

ASCORBIC ACID	NO. OF ANIMALS	SURVIVORS	AV. GAIN
mg/100 gm			*gm/day*
	With depletion [1]		
0.5	10	7	5.2 ± 0.7
0.4	10	8	4.7 ± 0.7
0.3	10	4	4.8 ± 0.4
0.2	10	2	3.9 ± 0.6
	Without depletion [2]		
0.4	6	6	4.1 ± 0.9
0.3	6	5	3.4 ± 0.7
0.2	6	6	2.6 ± 1.1

[1] Animals supplemented after 11 days of depletion for 80 days. Average gain includes the gains of the survivors from the 28th day through the 91st day.

[2] Animals supplemented from the beginning of the experiment for 35 days. Average gain represents the entire 5 week period.

slightly better. In the depletion experiment, symptoms of scurvy were observed only with 0.2 and 0.3 mg levels. No symptoms were found when the animals were supplemented from the beginning of the experiment. A comparison of the growth rates shown in table 2 indicates that the young animals grew at a faster rate after having been subjected to the stress of depletion.

The results of the tissue analyses are summarized in table 3. Between the lowest and the highest levels of ascorbic acid supplementation, there was a three-fold increase in the amount of ascorbic acid stored by the liver and spleen and a 5-fold

increase in the case of adrenals. No significant differences were observed in blood ascorbic acid concentrations. There were significant differences among all levels of ascorbic acid in liver, adrenals and spleen except between the 0.5 and 0.7 mg levels.

Although the growth rates in experiment 3 were slightly higher than those observed in experiment 2, the same trend was observed in that the 0.5 mg level gave an optimal growth rate of 5.6 gm per day.

TABLE 3

Tissue ascorbic acid concentration

ASCORBIC ACID	AV. GAIN [1]	LIVER	SPLEEN	ADRENAL
mg/100 gm	*gm/day*	*mg %*	*mg %*	*mg %*
0.2	3.4 ± 0.5 [2]	0.94 ± 0.20	5.38 ± 0.53	8.3 ± 0.8
0.3	4.0 ± 0.6	1.22 ± 0.16	6.86 ± 0.58	15.11 ± 2.11
0.4	4.6 ± 0.4	1.88 ± 0.14	9.62 ± 0.66	18.87 ± 0.96
0.5	5.7 ± 0.7	2.80 ± 0.31	14.02 ± 1.34	33.92 ± 5.63
0.7	5.1 ± 1.3	2.75 ± 0.56	14.97 ± 2.22	41.64 ± 6.76

[1] Average gain includes the period from the 28th day through the 49th day.
[2] Standard error.

DISCUSSION

Using a nutritionally adequate diet, growth appears to be a satisfactory criterion for determining the ascorbic acid requirement of the guinea pig. Although the optimal growth of the animals in our experiments is not as high as that reported by Reid and Briggs ('53), the consistent gain and the healthy state of the animals seems to make weight gain a satisfactory criterion. The minimum requirement for ascorbic acid based on growth, according to our experiments, is 0.5 mg per 100 gm of body weight per day. This is in line with the observation of Coward and Kassner ('36) that 1.0 mg is adequate for 250 gm animals. This also confirms the work of Dann and Cowgill ('36) and of Crampton et al. ('44) that the optimal level was 0.5 mg of ascorbic acid per 100 gm of body weight. Pfander and Mitchell ('53) suggest 0.7 mg per 100 gm per day when odontoblast length is the criterion. Using sub-

optimal diets, other workers have obtained values lower than 0.5 mg per 100 gm of body weight; they range from 0.16 to 0.4 mg per 100 gm (Waugh and King, '32; Dawborn, '45; Coward and Kassner, '36; Dunker et al., '42).

A correlation was observed between growth and tissue concentration of ascorbic acid. In the liver, spleen and adrenal glands, the concentration of ascorbic acid showed the same trend as the dose of vitamin C was increased. This relationship held within the range in which growth was proportional to ascorbic acid intake. Penney and Zilva ('46) observed that oral doses of ascorbic acid above a certain limit (0.5 to 1.0 mg) were only partially absorbed. Our results also suggest that at the highest level of supplementation absorption of the vitamin was restricted since the 0.7 mg per 100 gm level resulted in no increase in tissue ascorbic acid levels above those obtained with 0.5 mg.

The values obtained for ascorbic acid concentration of spleen and adrenals examined in this experiment are almost double those found by Kuether et al. ('44) for similar intakes of ascorbic acid up to the 0.5 mg level. This discrepancy in tissue concentration may be attributed to differences in the amount of the vitamin available to the animal. Kuether and co-workers added the vitamin to the diet and calculated the intake from food consumption data.

SUMMARY

The ascorbic acid requirement of guinea pigs fed a purified diet was determined using growth as the criterion. Depleted and non-depleted animals were used. Graded levels up to the 0.5 mg per 100 gm body weight level gave significant differences in growth during a 42-day period. The ascorbic acid requirement, according to our investigation, is 0.5 mg per 100 gm of body weight per day for both young and young adult animals.

Ascorbic acid was determined in spleen, liver, adrenals and blood 20 to 24 hours after supplementation. Significant differences were observed in the tissue ascorbic acid concentration

up to the 0.5 mg level of supplementation which gave maximum growth; no significant difference was observed between the 0.5 and 0.7 mg levels. This, too, tends to indicate a requirement of 0.5 mg per 100 gm of body weight. In blood, no significant differences among levels were observed.

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RELATION OF SATURATED, MEDIUM- AND LONG-CHAIN TRIGLYCERIDES TO GROWTH, APPETITE, THIRST AND WEIGHT MAINTENANCE REQUIREMENTS

HANS KAUNITZ, CHARLES A. SLANETZ AND RUTH ELLEN JOHNSON
Department of Pathology and the Institute of Comparative Medicine, Columbia University, New York, N. Y.
AND
VIGEN K. BABAYAN AND GEORGE BARSKY
Research and Development Laboratories of E. F. Drew and Co., Inc., Boonton, N. J.

(Received for publication July 17, 1957)

Information about the biological effects of medium-chain saturated fatty acids (6 to 12 carbons) and their triglycerides is sparse. The few studies carried out have dealt with their effect in vitamin B deficiency (Salmon and Goodman, '37), with their intestinal absorption (Bloom et al., '51), with how their oxidation differs from that of long-chain acids (Weitzel et al., '50) and with their effect upon the liver in choline deficiency (Gey et al., '55). Recently, some data on growth and serum cholesterol levels and on long-term studies indicating no toxic effects have been reported (Kaunitz et al., '58).

In the current studies, effects of diets containing triglycerides of medium-chain saturated fatty acids (MCT) and of long-chain saturated fatty acids (LCT) were compared with one another and with those of diets containing lard or no fat.

MATERIALS AND METHODS

The MCT was derived from coconut oil by fractionation of the split fatty acids and reconstitution of the desired fraction (6 to 12 carbon chains) into triglycerides. It represented about 15% of the original coconut oil. The oil was a clear, thin,

odorless liquid with a melting point below 0°C and an iodine number of less than one. The LCT was derived from coconut or palm kernel oils by hydrogenation of the fatty acids of 14 to 18 carbons and their reconstitution into triglycerides. The melting point of this material was about 40°C; its iodine number was 3 to 5.

These products or fresh lard were included at levels of 8 to 33% in a purified diet containing 30% alcohol-washed, vitamin-free test casein [1] (containing a maximum of 0.5% fat), 4% salts,[2] 2% cellulose [3] and dextrose to make 100%: To each kilogram of diet were added 1 gm choline, 1 gm inositol, 300 mg para-aminobenzoic acid, 100 mg nicotinamide, 10 mg vitamin K,[4] 2 mg thiamine, 4 mg riboflavin, 4 mg pyridoxine, 10 mg calcium pantothenate, 2.5 mg folic acid, 25 μg biotin, 5 mg 0.1% trituration of vitamin B_{12}, and 25 mg ascorbic acid. To each kilogram were also added 50 mg alpha-tocopherol acetate, 10 mg free alpha-tocopherol, 500 μg vitamin D_2, and 5 mg beta-carotene suspended in ml of MCT or linoleic acid.[5] When desired, a linoleic acid concentrate from safflower oil, containing at least 90% linoleic acid, was added (0.1 to 2.0%, always with the fat-free diets).

The experiments were carried out on male albino rats of the Sherman strain, which had been transferred at weaning (25 to 28 days) to a purified diet containing 30% lactalbumin and 10% lard. The technique of distribution into well-matched groups has been described before (Kaunitz et al., '54). At about 40 days, they were transferred to the experimental diets.

The methods of measuring food and water intakes for weight maintenance and the calculation of the growth requirements

[1] General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] U.S.P. XIII.

[3] Alphaeel, Nutritional Biochemicals Corp., Cleveland, Ohio.

[4] Synkayvite, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

[5] We are grateful to Dr. Leo A. Pirk of Hoffmann-LaRoche, Inc., Nutley, N. J., for most of the synthetic vitamins. We also wish to thank Barnett Laboratories, Long Beach, Calif., for the crystalline beta-carotene and Dr. M. L. Tainter of the Sterling-Winthrop Research Institute, Rensselaer, N. Y., for the crystalline vitamin D_2.

derived from these data have been reported recently (Kaunitz et al., '57a). For the calculation of the Caloric values of the diets, it was assumed that carbohydrate and protein yield 4.0 Cal. per gram and natural fats and LCT, 9.2 Cals. Bomb calorimetry gave a value of 8.3 Cal. per gram for MCT.

For the statistical analysis of the data the standard deviation, SD, was calculated according to the formula $\frac{\Sigma d^2}{n-1}$; the standard error, SE, was calculated by the expression $\frac{SD}{n}$. For the standard error of the difference between means: $SE_{m_1m_2} = \sqrt{SE_1^2 + SE_2^2}$. The means are significantly different ($P = 0.01$) if the "t" value (the difference of the two means divided by the SE of the difference) is three or greater. Some significance ($P = 0.05$) can be assumed if the "t" values are greater than two in repeated experiments.

EXPERIMENTS

In table 1 are given data as to growth, food intake, and water consumption of animals on diets containing 8 to 33% fat or no fat supplemented with linoleic acid. Body weights were highest for animals on lard diets and significantly lower on all other diets in any given series. The Caloric intake of freely eating animals on MCT was, in all three instances, somewhat higher than that of the lard-fed animals, but the differences were statistically not significant. Similar results as to food intake and growth were observed with animals on fat-free diets. The weights of the LCT groups were lowest, as were their Caloric intakes.

Fecal fat losses were not responsible for the reduced growth rate of the animals on MCT and LCT. When food consumption and, therefore, fat intake were measured for a certain period and the feces of the same period were analyzed for fat, the fecal fat contents with lard, MCT and LCT were found to be similar and not to exceed 2% of the ingested fat, as has been reported (Kaunitz et al., '57b). The high intestinal absorption of MCT is in good agreement with the results of Salmon and Goodman ('37).

TABLE 1

Average body weights, total Calorie intake and water consumption of rats fed, ad libitum, fat-free diets or those containing triglycerides of medium-chain saturated fatty acids (MCT), long-chain saturated fatty acids (LCT) or lard. (The diets with fat contained 0.1% linoleic acid)

SERIES	DIET	DURATION	NO. OF RATS	AV. BODY WEIGHT	"t" VALUE [1]	TOTAL CALORIC INTAKE	AV. PROTEIN INTAKE	Av. NaCl INTAKE	AV. WATER INTAKE	"t" VALUE [1]
		weeks		*gm*			*gm*	*gm*	*ml*	
D	20% Lard	7	7	297 ± 25 [2]		2656 ± 131	166	0.83	827 ± 55	
D	20% MCT [3]	7	7	252 ± 28	2.7	2736 ± 270	177	0.88	945 ± 80	3.0
C	30% Lard	5	6	275 ± 27		1975 ± 160	112	0.56	516 ± 38	
C	30% MCT	5	6	235 ± 9	3.4	1990 ± 232	118	0.59	595 ± 94	2.0
C	30% LCT [4]	5	6	194 ± 18	6.0	1640 ± 195	92	0.46	406 ± 57	3.9
C	Fat-free + 2% LA [5]	5	6	247 ± 11	2.3	2040 ± 67	159	0.79	605 ± 50	3.5
C	Fat-free + 0.3% LA	5	7	229 ± 19	2.9	1985 ± 123	158	0.80	578 ± 94	1.6
E	20% Lard	4	8	264 ± 13		1655 ± 105	104	0.52	584 ± 132	
E	20% MCT	4	8	231 ± 14	5.1	1690 ± 143	109	0.55	600 ± 204	0.2
E	20% LCT	4	7	229 ± 19	4.3	1575 ± 134	98	0.49	455 ± 106	2.2
F	8% Lard	6	8	258 ± 36						
F	8% MCT	6	8	224 ± 13	2.4					
F	Fat-free + 2% LA	6	8	230 ± 18	1.8					
F	33% Lard	6	8	270 ± 25						
F	33% MCT	6	8	220 ± 13	4.1					

[1] The "t" values refer to the lard animals of the same series.

[2] Standard deviation.

[3] Triglycerides of medium-chain saturated fatty acids.

[4] Triglycerides of long-chain saturated fatty acids.

[5] Linoleic acid concentrate.

In three experimental series, the average water intake of the animals on MCT was higher than for those on lard. In two instances, the "t" values were 2.0 and 3.0 and once, 0.2, when the individual variations were very high. These results indicate that the animals on MCT drank significantly more than did those on lard. In that the differences in Caloric, protein, and salt intakes were hardly sufficient to explain the increased water intakes, MCT must have been responsible. The water intakes of the freely-eating animals on fat-free diets were similar to those on MCT, although their protein and salt intakes were considerably higher.

With LCT, the water intakes were significantly lower than with lard. However, inasmuch as these animals ate less than those on lard, it is difficult to decide whether the decreased water intake was only due to a decreased food intake or was also caused by the fat itself. The fact that, in one series, the Caloric intake of the LCT animals was only about 2% less than that of the lard-fed animals whereas the water intakes differed by about 20% may be suggestive that LCT has an effect opposite to that of MCT.

In figure 1 are recorded results of experiments in which the animals' weights were kept constant within $\pm$ 2 gm by daily restricted feeding. The requirements are expressed as Calories per gram of body weight per week. Both the animals on MCT and LCT needed significantly more Calories for weight maintenance than those fed lard. In view of the fact that fat losses in the feces can be excluded as a source of the difference and that the Caloric value of MCT was checked by bomb calorimetry, it can be concluded that both LCT and MCT induce higher maintenance requirements than does lard. In previous experiments (Kaunitz et al., '57b), it was shown that the requirements of animals on 20% MCT were similar to those of rats on a fat-free diet.

In table 2 are summarized the water requirements of animals kept at constant weight. The animals on lard drank less than all other groups, which may have been partly related to the increased Caloric requirements of the latter groups. However,

proportionately, the water intake of those on MCT seemed to be higher than the Caloric differences. The average intake of the animals on MCT was in both experimental series, higher than that on LCT in spite of similar Caloric intakes, but the

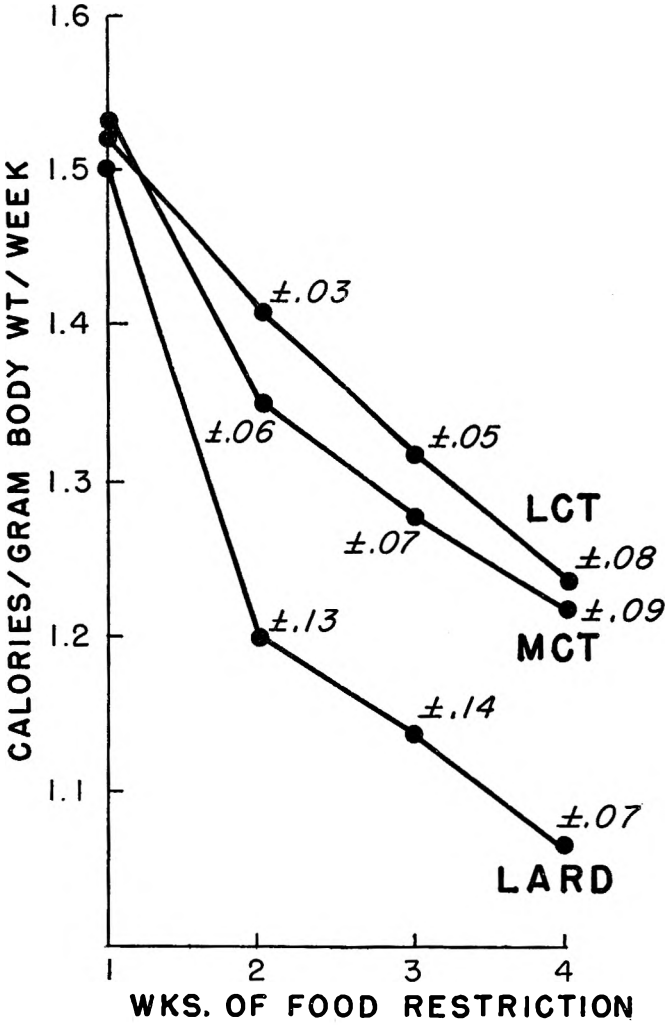

Fig. 1 Average Caloric requirements for weight maintenance of male rats (8 per group) on diets with 20% fat. The numbers next to the points are standard deviations. The probability factors for the LCT animals in comparison to those on lard were, from the second week, 3.3, 3.2, and 4.5; those for the MCT animals, 3.1, 2.7, and 3.7.

TABLE 2

Total water consumption of male rats of approximately 100 gm whose weight had been kept constant by restricted feeding (The diets with fat contained 0.1% of linoleic acid.)

SERIES	DIET	DURATION	NO. OF RATS	AV. WATER INTAKE	"t" VALUE	AV. CALORIC INTAKE	AV. PROTEIN INTAKE	AV. NaCl INTAKE
		weeks		*ml*			*gm*	*gm*
A, E	20% Lard	4	15	225 ± 53 [1]		572	36	0.18
A, E	20% MCT [2]	4	13	273 ± 65	2.2	647	42	0.21
A, E	20% LCT [3]	4	6	268 ± 35	2.2	610	38	0.19
C	30% Lard	5	7	203 ± 23		625	35	0.18
C	30% MCT	5	7	247 ± 47	2.2	645	38	0.19
C	30% LCT	5	7	225 ± 71	0.8	660	37	0.19
C	Fat-free + 0.3% LA [4]	5	7	241 ± 19	2.9	642	51	0.26
C	Fat-free + 2% LA	5	7	262 ± 48	3.0	635	49	0.25

[1] Standard deviation.

[2] Triglycerides of medium-chain saturated fatty acids.

[3] Triglycerides of long-chain saturated fatty acids.

[4] Linoleic acid concentrate.

TABLE 3

Caloric requirements of realimented male rats with respect to body weight increase and weight maintenance after a period of restricted food intake

SERIES	DIET	NO. OF RATS	AV. BODY WT. AT END OF FOOD RESTRICTION	WT. INCREASE DURING 3 DAYS	TOTAL FOOD INTAKE DURING 3 DAYS	WEIGHT MAINTENANCE REQUIREMENTS	CALORIES PER GRAM BODY WT. INCREASE
			gm	*gm*	*gm*	*gm*	
A	20% Lard [1]	7	90 ± 4.1 [2]	27 ± 4.5	33.5 ± 1.3	10.1 ± 1.3	4.1 ± 0.3
A	20% MCT [3]	6	91 ± 4.0	21 ± 2.3	29.6 ± 1.3	12.8 ± 0.8	3.7 ± 0.5
A	20% MCT + 2% LA [4]	8	90 ± 1.7	23 ± 3.2	29.8 ± 2.7	11.8 ± 1.3	3.6 ± 1.2
C	30% Lard [1]	7	107 ± 3.4	27 ± 3.4	27.0 ± 2.7	10.7 ± 0.7	3.1 ± 0.4
C	30% MCT [1]	7	106 ± 3.9	26 ± 4.8	32.0 ± 7.2	11.8 ± 0.9	3.7 ± 1.1
C	30% LCT [1,5]	7	104 ± 1.5	19 ± 2.0	21.0 ± 2.7	11.4 ± 0.7	2.7 ± 1.6
C	Fat-free + 0.3% LA	7	105 ± 1.6	24 ± 2.0	32.0 ± 3.0	15.4 ± 0.5	2.6 ± 0.3
C	Fat-free + 2% LA	8	107 ± 2.2	25 ± 1.7	33.0 ± 1.6	16.0 ± 0.9	2.6 ± 0.6

[1] These groups received 0.1% of linoleic acid.

[2] Standard deviation.

[3] Triglycerides of medium-chain saturated fatty acids.

[4] Linoleic acid concentrate.

[5] Triglycerides of long-chain saturated fatty acids.

differences were not significant. As with the freely-eating animals, the water and Caloric intakes of the MCT animals and those on fat-free diets were similar.

In table 3 are given data as to the Caloric requirements for growth above the maintenance requirements. Animals maintained at constant weight were permitted to eat freely for three days, and their weight increases and food intakes were measured. As has been reported in detail (Kaunitz et al., '57a), these data can be used to calculate the requirements for one gram of body weight increase. It can be seen from table 3 that the requirements of the animals on lard and on MCT were similar. With LCT and fat-free diets, the requirements were lower. With LCT, this difference was, however, statistically not significant compared with MCT ($P = 1.3$). With both fat-free diets, the "t" values compared with MCT were 2.5 in both instances, and compared with lard, 2.6 and 1.9.

It has been shown (Hausberger, '37; Stoerk and Porter, '50) that the testicular fat body is a structure the weight and growth of which is as predictable as that of other organs. Furthermore, the weight changes of this body were found to be parallel to the content of neutral fat in the carcasses and livers of rats.

We found, on 100 male rats of more than 100 gm on diets containing 8 to 33% lard, that a log-log plot of the weight of testicular fat against the body weight resulted in a straight line with a slope of about 60° from the abscissa; the spread was approximately $\pm 20\%$.

In table 4 are presented weights of testicular fat body calculated per 100 gm of body weight This can be justified in view of the relatively narrow range of body weights, especially in the case of the animals whose weights had been kept constant. However, log-log plotting was also done (omitted for brevity), and inferences were drawn only when both methods indicated the same differences.

As indicated in the table, the fat bodies of freely-eating animals receiving LCT or lard are significantly heavier than those receiving either MCT or a fat-free diet. The groups on

fat-free diets receiving 0.3 and 2% of linoleic acid concentrate were identical and therefore combined. The fat bodies of the animals on MCT and fat-free diets were similar in weight. In animals on restricted food intake, the relationships of the fat bodies were comparable to those of the freely-eating ones. Again, the animals on lard and LCT had fat bodies of similar

TABLE 4

Average weight of testicular fat body per 100 gm of body weight in male rats fed fat-free diets or diets containing 30% fat

DIET	NO. OF RATS	AV. BODY WEIGHT	TESTICULAR FAT BODY WEIGHT PER 100 GRAMS	"t" VALUE
			gm	
		Freely eating		
Lard [1]	6	278	1.36 ± 0.22 [2]	
MCT [2]	7	235	0.84 ± 0.11	5.3 *
LCT [3]	6	195	1.05 ± 0.14	2.9 **
Fat-free [4]	13	237	0.87 ± 0.10	5.3 *
		Restricted		
Lard	7	135	0.82 ± 0.23	
MCT	7	132	0.55 ± 0.14	2.6 *
LCT	6	124	0.84 ± 0.20	3.0 **
Fat-free	15	131	0.63 ± 0.12	2.0 *

[1] The diets containing fat were supplemented with 0.1% of linoleic acid.
[2] Standard deviation.
[3] Triglycerides of medium-chain saturated fatty acids.
[4] Triglycerides of long-chain saturated fatty acids.
[5] The fat-free diets were supplemented with either 2 or 0.3% of linoleic acid.
* "t" value compared to lard.
** "t" value compared to MCT.

size, and those of the animals on MCT and fat-free diets were smaller. It can therefore be concluded that, when diets containing 30% or no fat are fed, more neutral fat is deposited in the animals on lard or LCT than in the rats on MCT or no fat.

Natural fats, such as lard, improve the efficiency of food utilization; this means that, on a fixed Caloric intake, a high-fat diet will lead to a higher weight than a fat-free or fat-low ration (Swift and French, '54). Similarly, it has been noted

that the Caloric requirements for weight maintenance are lower on a high-fat than on a high-carbohydrate diet (Kaunitz et al., '56). With MCT, the animals behaved more like those on a fat-free diet.

MCT was used on two humans who ate, during 4 consecutive weeks, a diet containing 1400 Cal. with MCT as the source of fat in the first and third and butter and other fats in the second and 4th weeks. The weight losses ranged, during the MCT periods, from 4 to 8 lbs. and, during the butter periods, from 0 to 4 lbs. MCT was later used on 20 obese persons without control periods. Weight losses of up to 30 lbs. in two months were observed; some substantial losses were noted in persons who had previously tried to lose weight on diets with comparable Caloric contents. However, no claims should be made as to the clinical usefulness of this tailor-made fat.

SUMMARY

1. Triglycerides of saturated, medium-chain (6 to 12 carbons) fatty acids (MCT) and of saturated, long-chain (14 to 18 carbons) acids (LCT), which had been prepared from coconut and other palm kernel oils were studied in feeding experiments on rats receiving a purified diet with 30% casein. Their effects on growth, food and water intakes, weight maintenance requirements, and the testicular fat body were compared with those of lard and fat-free diets.

2. The body weights of freely-eating rats on lard were significantly higher than those of comparable animals on MCT, the latter being similar to those on fat-free diets. Those on LCT grew least.

3. The water consumptions of the rats on MCT were the highest; the Caloric intakes of those on the MCT and fat-free diets were similar and, on the average, slightly higher (not significantly) than that of the lard animals. The LCT animals ate and drank least.

4. The weight maintenance requirements of animals kept at constant weight by restricted feeding were significantly

higher for the groups fed MCT and LCT than for the groups fed lard. Among the animals kept at constant weight, those on MCT and fat-free diets drank significantly more than those on LCT or lard.

5. The testicular fat bodies (which are roughly proportional to total neutral fat) were heaviest in relation to the body weight in animals on 30% lard and on LCT and lower for those on 30% MCT and fat-free diets. This was also true of animals on the same diets on restricted food intake.

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STUDIES RELATED TO "VITAMIN B_{13}"

WALTHER H. OTT, ALICE M. DICKINSON AND ANN VAN INDERSTINE
Merck Institute for Therapeutic Research
AND
ALVA W. BAZEMORE, ATWOOD C. PAGE, JR. AND KARL FOLKERS
Merck Sharp and Dohme Research Laboratories, Rahway, N. J.

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In the continuing search for nutritional factors of importance in animal nutrition, we have, over the past few years, performed a number of experiments related to "vitamin B_{13}." In a recent paper from this laboratory (Skeggs, Wright, Cresson, MacRae, Hoffman, Wolf and Folkers, '56) literature on "vitamin B_{13}" has been reviewed.

The name "vitamin B_{13}" was applied by Novak and Hauge ('48) to a material which they isolated from distillers' dried solubles. They had previously reported that distillers' dried solubles contained an unidentified factor or factors necessary for the growth of chicks (Novak, Hauge and Carrick, '47). Subsequently, Rasmussen, Luthy, Van Lanen and Boruff ('54) described a new basal ration for measuring "vitamin B_{13}" activity with chicks.

In view of these reports, investigations of the postulated factor were undertaken. Although the results obtained with chicks were equivocal or negative, we believe that a brief report of the findings would be of value.

RESULTS AND DISCUSSION

Novak and Hauge ('48) have described in detail a procedure for the purification of "vitamin B_{13}" from distillers' dried solubles, liver and rice polishings. Several 100-pound samples of distillers' dried solubles were processed in this laboratory as nearly as possible by the described procedure.

The distillers' dried solubles were subjected to the hydrolysis, alcohol precipitation and pH precipitation steps. The first sample (fraction 1, table 1) was taken at that stage and concentrated to a low volume to facilitate addition to the chick diet. After the removal of material by fullers' earth adsorption, samples of the filtrate were similarly prepared for assay (fraction 2, table 1). Precipitation with phosphotungstic acid, removal of the phosphotungstic acid as the barium salt, and subsequent chloroform extraction of an aqueous solution of the material gave rise to the chloroform soluble extractives (fraction 3, table 1). Fractions 2′ and 3′ were prepared by the same process as fractions 2 and 3, but were made from different lots of starting material.

Day-old New Hampshire chicks obtained from a commercial hatchery were used in these experiments. The chicks were fed basal ration for three days and then distributed into experimental groups of 10 chicks each equally balanced on the basis of individual body weights. The diets used were corn and soybean oil meal rations described in detail in table 3. It had been found in other experiments that the addition of a casein digest to the basic corn-soybean diet improved growth, although not to the same extent as fish meal. An example of this partial response is given with ration C in table 2. In addition to this, the casein hydrolysate was found by us to improve the response to certain fermentation products. In diets A and B, it was used in the diet in an attempt to provide the optimum conditions for a highly purified component to show biological activity.

In each of the experiments described in table 1, two groups of 10 chicks each received the samples and 4 groups of 10 chicks each constituted the positive and negative controls. The first two columns of chick data were obtained with fractions from one batch of distillers' solubles. The third and 4th columns were both obtained with fractions from another batch.

TABLE 1

Data from tests of "vitamin B_{13}" concentrates in chicks

SUPPLEMENT	AMOUNT PER KG DIET	EQUIVALENCE [2] TO DDS	WEIGHT GAIN TO 3 WEEKS OF AGE [1] AVERAGE ± STANDARD ERROR				RELATIVE GAIN OVER APPROPRIATE BASALS
			Diet A	Diet B			
				Exp. 1	Exp. 2	Exp. 3	
		gm	*gm*	*gm*	*gm*	*gm*	*av. %*
Basal (none)			148 ± 4 (4)	185 ± 7 (4)	184 ± 7 (4)	175 ± 5 (4)	0
Fraction 1	22 gm	100	152 ± 6 (2)				+ 3 [3]
Fraction 2	18 gm	200		188 ± 10 (2)			+ 2
Fraction 2'	—	400			180 ± 9 (2)		— 2
Fraction 3	60 mg	200		182 ± 10 (2)			— 2
Fraction 3'	270 mg	400				182 ± 8 (2)	+ 4 [3]
Fish meal	100 gm		163 ± 4 (4)	211 ± 7 (4)	211 ± 7 (4)	201 ± 5 (4)	+ 13 [4]

[1] Numbers of groups of 10 chicks each are shown within parentheses.

[2] This was the amount of distillers' dried solubles fractionated to give rise to the amount of sample added to 1 kg of diet.

[3] Not significant; $P > 0.20$.

[4] Highly significant; $P < 0.01$.

No significant growth-promoting effects were observed in the groups receiving any of the distillers' dried solubles fractions which we have made according to the procedures described by Novak and Hauge ('48).

Samples of a preparation labeled "Vitamin B_{13}" concentrate and stated to have been made from distillers' solubles were kindly supplied to us by Drs. Leonard Stone and J. M. Van Lanen of Hiram Walker and Sons, Inc. Their concentrate was examined in chick experiments using several different diets. The results of these experiments are given in table 2. These experiments included tests of orotic acid since Manna and Hauge ('53) considered that orotic acid and "vitamin B_{13}" may be related. The diets used in these tests include the diets discussed in connection with table 1 and, in addition, a purified isolated soybean protein diet (ration D). Furthermore, we also employed a diet containing blood meal, ration E, as similar as possible to that used by Rasmussen, Luthy, Van Lanen and Boruff ('54) in their tests on "vitamin B_{13}" concentrates.

We have not examined the casein-wheat diets described by Novak, Hauge and Carrick ('47). The experiments described by these workers support the conclusion that the growth promoting factor in distillers' dried solubles and fish solubles is not identical with folic acid. However, the identity of the active factor with vitamin B_{12} cannot be excluded on the basis of the evidence presented in their publication.

Appraisal of the data in table 2 leads us to the opinion that the response elicited by the Hiram Walker concentrate, although tending to be positive, is far from being large enough to be statistically significant in this limited series of tests. Certainly, the animals receiving the concentrate did not grow as well as the animals receiving crude dietary supplements including distillers' dried solubles (table 2, ration E). However, it appears from the current literature that such crude materials may contain several unidentified growth factors—no one of which alone would be expected to give the full effect of the crude material.

TABLE 2

Chick tests on the Hiram Walker concentrate from distillers' solubles

RATION	SUPPLEMENT	WEIGHT GAIN TO 3 WEEKS OF AGE AVERAGE ± STANDARD ERROR				RELATIVE GAIN OVER CONTROLS
		gm				*av. %*
A	Basal (none)	175 ± 5 (4)				0
	Concentrate, 50 mg/kg diet	181 ± 7 (2)				+ 4 [1]
	Fish meal, 10%	181 ± 5 (4)				+ 4 [1]
		EXP. 1	EXP. 2	EXP. 3	EXP. 4	
B	Basal (none)	140 ± 5 (4)	151 ± 7 (2)	109 (1)	152 (1)	0
	Concentrate, 50 mg/kg	132 ± 5 (4)	—	126 (1)	151 (1)	— 1
	Orotic acid, 100 mg/kg	129 ± 7 (2)	155 (1)	—	—	— 4
	Orotic acid, 100 mg/kg + concentrate, 50 mg/kg	—	—	128 (1)	140 (1)	+ 5 [1]
	Fish meal, 10%	174 ± 5 (4)	176 ± 7 (2)	136 (1)	181 (1)	+ 21 [2]
		EXP. 1		EXP. 2		
C	Basal (none)	138 ± 4 (2)		132 ± 4 (2)		0
	Casein hydrolysate,[3] 10%	—		142 ± 4 (2)		+ 8 [4]
	Casein hydrolysate, 10% + concentrate, 50 mg/kg	160 ± 4 (2)		—		+ 16 [4]
	Fish meal, 10%	160 ± 4 (2)		161 ± 4 (2)		+ 19 [2]
D	Basal (none)	154 ± 7 (4)				0
	Concentrate, 50 mg/kg	160 ± 10 (2)				+ 4 [1]
	Fish meal, 10%	170 ± 7 (4)				+ 10 [1]
E	Basal (none)	93 ± 3 (6)				0
	Concentrate, 30 mg/kg	92 ± 3 (6)				— 1
	Fish solubles, 5%	105 ± 3 (6)				+ 13 [2]
	Distillers' dried solubles,[5] 10%	129 ± 3 (6)				+ 39 [2]

[1] Not significant; $P > 0.10$.
[2] Highly significant; $P < 0.01$.
[3] N-Z-Amine type E, Sheffield Chemical Co., Division National Dairy Products Corp.
[4] Significant; $P = 0.01$.
[5] Stimufiav, Hiram Walker & Sons, Inc.

TABLE 3

Composition of chick rations

RATION	A	B	C	D	E	
	gm	*gm*	*gm*	*gm*		*gm*
Sucrose	—	—	—	57.8	Glucose (cerelose)	57.7
Yellow corn meal	58.2	58.2	58.2	—	Blood meal	17.5
Soybean oil meal	32.0	32.0	32.0	—	Soybean meal [1]	14.1
Soybean protein [2]	—	—	—	22.0	Gelatin	3.5
Alfalfa meal	—	—	5.0	—	Corn oil (Mazola)	0.25
Casein hydrolysate [3]	5.0	5.0	—	—	Choline chloride	0.8 [4]
Cellulose [5]	—	—	—	5.0	DL-methionine	0.2
Soybean oil	—	—	—	3.0	Vitamin A and D mix [6]	0.15
Liver fat [7]	—	—	—	3.0	Diphenyl-*p*-phenylenediamine	0.05
Glycine	—	—	—	1.0	Vitamin E mix [8]	0.045
						mg
DL-methionine	0.1	0.1	0.1	0.75	Inositol	10.0
Choline chloride	0.4 [4]	0.4 [4]	0.4 [4]	0.2	Ascorbic acid	10.0
Vitamin A and D mix [6]	0.2	0.2	0.2	0.1	Niacin	3.2
	mg	*mg*	*mg*	*mg*		
Inositol	50.0	50.0	50.0	100.0	Thiamine	2.0
p-aminobenzoic acid	15.0	15.0	15.0	30.0	Calcium pantothenate	2.0
Niacin	2.0	2.0	2.0	10.0	Riboflavin	0.70
Calcium pantothenate	1.5	1.5	1.5	4.0	Pyridoxine	0.70
						mg
Pyridoxine	0.5	0.5	0.5	2.0	*p*-aminobenzoic acid	0.20
Riboflavin	0.5	0.5	0.5	2.0	Folic acid	0.14
Thiamine	0.25	0.25	0.25	2.0	Menadione	0.10
Menadione	0.05	0.05	0.05	0.4	Biotin	0.002
Biotin	0.0125	0.0125	0.0125	0.04	Vitamin B_{12}	0.002
						gm
Vitamin B_{12}	0.005	0.005	0.005	0.005	Steamed bonemeal	3.45
Folic acid	—	—	—	0.4	$CaHPO_4 \cdot 2H_2O$	1.10
α-tocopherol	—	—	—	0.4	NaCl	0.65
Procaine penicillin [9]	—	1.0	—	—	$K_2CO_3 \cdot 1.5H_2O$	0.20
				gm	KCl	0.20
Salt mix [10]	—	—	—	5.0		
Bone ash	—	—	—	2.0		*mg*
	gm	*gm*	*gm*		$MnSO_4$ [9]	40.0
Steamed bonemeal	2.0	2.0	2.0	—	Iron ammonium citrate	21.0
Ground limestone	1.5	1.5	1.5	—	$MgCO_3$	15.0
Sodium chloride	0.5	0.5	0.5	—	$ZnCl_2$	2.0
Manganese sulfate [9]	0.02	0.02	0.02	—	$CuSO_4$	0.82
					KI	0.20
					H_3BO_3	0.085
					$CoSO_4 \cdot 7H_2O$	0.050

[1] Nutrisoy (50% protein), Archer-Daniels-Midland Co.
[2] C-1 Assay Protein, The Drackett Co.
[3] N-Z-Amine type E.
[4] As 25% dry mix.
[5] Celluflour, Chicago Dietetic Supply House
[6] Viadex (4000 A, 750 D per gram), National Oil Products Co.
[7] Lipid material extracted from liver with halogenated solvent, Viobin Corp.
[8] Myvamix (44 units E per gram), Distillation Products Industries.
[9] Feed grade.
[10] Hegsted et al. ('41).

SUMMARY

Under the specific dietary conditions reported herein, no significant growth-promoting effect in chicks was observed with either "vitamin B_{13}" concentrates prepared from distillers' solubles by the method of Novak and Hauge, or a "vitamin B_{13}" concentrate prepared in another laboratory.

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EFFECTS OF THE PREVENTION OF COPROPHAGY IN THE RAT

I. GROWTH STUDIES [1]

RICHARD H. BARNES AND GRACE FIALA
Graduate School of Nutrition, Cornell University, Ithaca, New York

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A new technique for the complete prevention of coprophagy in the rat has been described by Barnes et al. ('57). Utilizing this procedure, an indirect measurement indicated that the rat maintained on a raised wire screen ingests approximately 50% of its excreted feces. This relatively large recycling of fecal material suggests that coprophagy may have an important role in the nutrition of the rat. Several studies have provided evidence that the rat can acquire a significant portion of its nutritive requirements by fecal ingestion (Steenbock et al., '23; Barki et al., '49) and one report (Geyer et al., '47) indicates that when coprophagy is completely prevented there may be a requirement for an unidentified growth factor.

The present study is a general survey of crude natural materials that frequently have been suggested as sources for unidentified factors for growth. The purpose has been to explore the possibility that some unknown growth factor is required by the rat when coprophagy is prevented.

METHODS

The procedure for the prevention of coprophagy has been described previously (Barnes et al., '57). In brief, a plastic cup, shaped so as to cover the anus and prevent access to

[1] This research has been supported in part by a grant from The National Science Foundation.

the feces as they are extruded, is slipped over the tail and held in place by a felt collar that is wrapped around the tail. This cup permits free movement by the rat, but obviously is a source of annoyance, as evidenced by the rat's persistent efforts to dislodge it. This attitude is most noticeable during the first half hour after the cup is attached. However, certain rats, particularly when fed a deficient diet, chew at the rubber sleeve covering and any exposed areas of plastic with fantastic perseverance. Rats that are maintained on a normal, complete diet are less obviously annoyed by the cup and are maintained for 4 to 6 weeks without difficulty.

All rats used in this study were males obtained at weaning age.[2] When received in the laboratory they were maintained on the control diet (table 1) for three days, then ear-marked, weighed, and separated into groups of 10 rats each so as to randomize body weight. They were housed in individual wire screen-bottom cages in a room maintained at 74° ± 2°C and fed diets ad libitum. Feces were emptied from the cups daily and the rats were weighed weekly. Except where noted in table 2, growth results have been tabulated for 28 days.

The composition of the control diet is shown in table 1. Succinylsulfathiazole was added to the complete diet. All other additions were made at the expense of the cerelose content. Butter fat replaced the commercial hydrogenated vegetable oil [3] in equal amounts and the low-fat diet was made by removing the hydrogenated vegetable oil entirely and replacing it with an equal quantity of cerelose. A small amount of water (approximately 5%) was added to the low-fat diet in order to improve its consistency.

RESULTS AND DISCUSSION

Succinylsulfathiazole. In every instance a growth depression was noted when coprophagy was prevented. This was always associated with a decreased food intake. There is some indication that with added experience in the manipula-

[2] Holtzman, Madison, Wisconsin.

[3] Primex, Procter and Gamble, Cincinnati, Ohio.

tion of the feces cups this growth inhibition was lessened. (Compare the more recent studies — experiments 11 and 16, with earlier ones — experiments 4 and 7). Succinylsulfathiazole was added to the diet in anticipation of a further growth retardation due to inhibition of intestinal synthesis of essential nutrients. This appeared to be the case in the earlier studies (experiments 5 and 8), but was not borne out later (experiments 10 and 11). An unusually severe diarrhea developed in all rats receiving the sulfonamide which finally disappeared after approximately two weeks. The addition of bulk in the form of ground cellophane was fairly effective in preventing this.

TABLE 1

Diet Composition for 100 gm

MAJOR COMPONENTS	
	gm
Casein [1]	25.0
Cerelose	53.0
Primex [2]	15.0
Salts [3]	4.0
Choline dihydrogen citrate	0.3
B vitamins in sucrose	2.0
Fat soluble vitamins in corn oil	1.0
	100.3

B VITAMINS IN 2.0 GM SUCROSE		FAT SOLUBLE VITAMINS IN 1.0 GM CORN OIL	
	mg		*mg*
Thiamine HCl	0.40	Vitamin A acetate	0.31
Riboflavin	0.80	Vitamin D (calciferol)	0.0045
Pyridoxine HCl	0.40	Alpha tocopherol	5.00
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		

[1] Vitamin test, General Biochemicals, Inc.

[2] Proctor and Gamble.

[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273, 1937.

TABLE 2

The effect of dietary supplements upon the growth of rats in which coprophagy has been prevented

EXPERIMENT NO.	TREATMENT	DAILY GAIN BODY WEIGHT	CHANGE FROM CONTROL
		gm	%
4 (31 days)	Control	6.3	Control
	Control + feces cups	5.3	— 18
	Control + 2% SST [1]	6.5	Control
	Control + 2% SST + feces cups	4.7	— 28
5 (31 days)	Control + 2% SST	6.2	Control
	Control + 2% SST + feces cups	5.0	— 19
	Control + 2% SST + feces cups + whey [2] (5%)	5.0	— 19
	Control + 2% SST + feces cups + D.D.S.[3] (5%)	4.7	— 24
	Control + 2% SST + feces cups + fish sol.[4] (5%)	4.8	— 23
7 (28 days)	Control	5.9	Control
	Control + feces cups	4.6	— 22
	Control + feces cups + cellophane (5%)	5.0	— 15
	Control (butter fat)	5.8	Control
	Control (butter fat) + feces cups	4.6	— 21
8 (29 days)	Control + 2% SST + 3% cellophane	6.0	Control
	Control + 2% SST + 3% cellophane + feces cups	4.7	— 22
	Low fat	5.4	Control
	Low fat + feces cups	4.7	— 13
	Low fat + 2% SST + 3% cellophane	5.5	Control

9 (28 days)	Control (20 rats)	[illegible]	[illegible]
	Control + feces cups	5.6	— 14
	Control + feces cups + feces[5] (5%)	5.0	— 23
	Control + feces cups + feces[6] (5%)	4.7	— 28
10 (28 days)	Low fat + 2% SST + 3% cellophane	5.5	Control
	Low fat + 2% SST + 3% cellophane + feces cups	5.0	— 9
	Low fat + 2% SST + 3% cellophane + feces cups + liver[7] (5%)	4.7	— 14
11 (28 days)	Control	6.6	Control
	Control + feces cups	5.9	— 11
	Control + feces cups + feces[8]	5.2	— 21
	Low fat + 2% SST + 3% celluflour[9]	5.8	Control
	Low fat + 2% SST + 3% celluflour + feces cups	4.8	— 17
	Low fat + 2% SST + 3% celluflour + feces cups + feces[8]	4.8	— 17
16 (28 days)	Control	6.4	Control
	Control + feces cups	5.6	— 12
	Control + 3% celluflour	6.5	Control
	Control + 3% celluflour + feces cups	5.7	— 12
	Control + 10% celluflour	6.1	Control
	Control + 10% celluflour + feces cups	5.3	— 13

[1] Succinylsulfathiazole.
[2] Dried whey.
[3] Distillers' dried solubles.
[4] Condensed fish solubles.
[5] Feces from control rats lyophilized and added to diet.
[6] Feces from feces cups lyophilized and added to diet.
[7] Viobin defatted liver powder.
[8] Fresh feces from cups put in separate feeder daily.
[9] Chicago Dietetic Supply Co.

Whey, dried distillers' solubles, fish solubles and liver. These natural materials have been suggested in numerous studies as possible sources of unidentified nutrients. In addition, Geyer et al. ('47), found liver to be effective in combatting the growth depression of rats in which coprophagy was prevented. In the present studies none of these supplements improved the growth rate of coprophagy-prevented rats (experiments 5 and 10).

Fat. In studies with mice, Bosshardt et al. ('50) found evidence of an unidentified nutrient requirement when low-fat diets, combined with succinylsulfathiazole were fed. With the various supplements studied here there was no clear indication of an added growth requirement when fat was restricted and coprophagy prevented, but the characteristic growth depression due to fat restriction *per se* was noted. In the low-fat diets, 1% of corn oil containing the fat-soluble vitamins was included. The addition of succinylsulfathiazole to the low-fat diets did not appear to accentuate growth depression when coprophagy was prevented. In experiment 8, a 24% decrease in weight gain was noted with these dietary conditions, but this was greater than was observed in any subsequent experiment, and it is believed that this one example does not warrant a conclusion that low-fat, succinylsulfathiazole-containing diets accentuate growth depression in the rat which does not have access to its feces.

Geyer et al. ('47) found that butter fat used in place of corn oil prevented the characteristic growth depression in their rats in which coprophagy had been prevented by holding the animals in a specially designed tubular cage. In the present series of experiments, the replacement of the hydrogenated shortening that was normally used in the diets by butter fat was without effect upon the growth rate of the rats in which coprophagy was prevented.

Undigestible residue. The addition of a small amount of finely ground cellophane to the diet which contained 2% of succinylsulfathiazole helped to control diarrhea in the rats that were prevented from eating their feces. In addition,

an early experiment (experiment 7) gave some indication that roughage in the diet may have stimulated growth in the rat in which coprophagy was prevented. Experiment 16 was set up to examine this point more thoroughly and, as can be seen, neither 3 nor 10% of roughage [4] had any effect upon growth rate.

Feces. The most objective test for the existence of an unidentified growth factor requirement in the rat in which coprophagy is prevented would seem to be the feeding of feces to such animals and observing the effect upon growth rate. The first attempt at this type of study was in experiment 9. Feces were collected from under the cages of the control rats or from the feces cups of rats from the second group in this experiment. Daily collections were frozen and when a sufficient supply had been collected they were dried by lyophilization. The dried feces were incorporated in the control diet at a level of 5%. Both groups of rats receiving the diets containing dried feces showed an additional growth depression. A second study (experiment 11) was then set up to examine the effect of presenting each rat with the fresh, daily collected feces from its own feces cup. These feces were placed in a separate jar and it was observed that the rats consumed a fairly large portion of feces that were offered. However, no stimulation of growth was noted either in the rats receiving the control diet or a low-fat diet with 2% of succinylsulfathiazole. In fact, in the first instance a growth depression was found.

The supplementation studies that are reported here fail to provide any evidence that feces contain an unknown growth factor and that the depression in growth resulting from the prevention of coprophagy is due to a nutritional deficiency. The most logical explanation for this growth inhibition would seem to be in terms of physiological (or psychological) strain that is imposed by the presence of the plastic cup and felt collar that is attached to the rat's tail. Although free movement is permitted, the feces cups must impose a continuing

[4] Cellu flour, Chicago Dietetic Supply House, Chicago, Illinois.

source of annoyance. This is not manifested to any noticeable degree by the rat consuming a completely adequate diet. Unfortunately, it is impossible to rule out entirely the possibility that some unidentified nutrient is lacking in the rats in which coprophagy is prevented. An exact control situation has not been provided in these experiments nor has such a procedure been devised. Free access to fecal pellets as they are excreted and at the same time having in place a sham feces collector would be required for optimal control.

SUMMARY

When coprophagy is prevented in the rat by the use of a plastic cup fitted over the tail, a depression in growth rate results. This growth retardation was not prevented by adding to the diet liver, whey, distillers' dried solubles, fish solubles, cellophane, or rat feces. Neither was the replacement of the fat of the diet by butter fat of any effect upon growth rate. Present evidence does not warrant a conclusion that an unidentified growth factor is required by the rat in which total prevention of coprophagy is maintained.

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METABOLISM OF CESIUM AND POTASSIUM IN SWINE AS INDICATED BY CESIUM-134 AND POTASSIUM-42 [1]

FRANK R. MRAZ, ANDREW M. JOHNSON
AND HOMER PATRICK
University of Tennessee — Atomic Energy Commission Agricultural Research Program, Oak Ridge, Tennessee

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Inasmuch as cesium is one of the more important long-lived fission products, there has been interest in obtaining data on the metabolism of this element in the animal body.

Cesium, as an alkali metal, would be expected to behave similarly to sodium, potassium and rubidium, chemically. Cesium and potassium are known to enter the solute complex, participating in ion antagonism, osmosis, permeability regulation, maintenance of the colloidal state and similar physiological phenomena (MacLeod and Snell, '50). Mraz et al. ('57) observed a decrease in uptake and an increase in excretion of cesium-134 by the rat when dietary potassium was increased. Hood and Comar ('53) reported that a Hampshire barrow excreted in 7 days 24.6% of the administered cesium-137 dose in the urine and 9.0% in the feces. Mraz and Patrick ('57) reported that supplementation of a simplified (casein-cornstarch) rat diet with some natural feedstuffs increased total excretion of cesium-134 and potassium-42 and decreased the ratio of urinary to fecal excretion of cesium-134 and potassium-42.

[1] This manuscript is published with the permission of the director of the University of Tennessee, Agricultural Experiment Station, Knoxville, Tennessee. The radioactive materials used in this work were obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission. This work was completed under contract no. AT-40-1-GEN-242 between the University of Tennessee, College of Agriculture, and the Atomic Energy Commission.

This study was initiated to ascertain whether total excretion and the excretory pattern of cesium-134 and potassium-42 in the pig could be influenced by natural feedstuffs or potassium in the ration.

EXPERIMENTAL

The three basal rations used during these studies are shown in table 1.

TABLE 1

Basal rations used in studies of the metabolism of cesium and potassium

INGREDIENTS	BASAL RATION		
	A	B	C [1]
Casein	..	5.0	20.0
Soybean protein [2]	10.0	10.0	..
Corn meal	63.2	35.2	..
Cornstarch	25.0	37.0	65.4
Cerelose	..	10.0	..
Wesson oil	..	..	5.0
Beet pulp	..	..	5.0
Dicalcium phosphate	1.0	1.0	1.0
Calcium carbonate	1.0	1.0	1.0
Sodium chloride	0.5	0.5	0.5
K. def. trace mineral mixture [3]	..	..	0.1
Vitamin concentrate	0.3 [4]	0.3 [4]	2.0 [5]

[1] Potassium content as determined by analysis of ingredients with a flame photometer is 0.035%.

[2] Drackett Products Company, Cincinnati, Ohio.

[3] Potassium-deficient trace mineral mixture used by Hove and Herndon ('55).

[4] Vitamin concentrate supplied the following amounts of vitamins per pound of ration. Vitamin A 2270 I. U., vitamin D 681 I. U., vitamin B_{12} 0.006 mg, riboflavin 2 mg, calcium pantothenate 4 mg, niacin 9 mg, choline chloride 10 mg and folic acid 0.06 mg.

[5] Vitamin concentrate differed from the above by containing an additional 2 mg calcium pantothenate, 6 mg niacin, 444 mg choline chloride, 1.44 mg folic acid, 1 mg thiamine, 1.5 mg pyridoxine and 4 mg penicillin per pound of ration.

In the first experiment, 12 pigs of mixed ancestry were divided in three groups of 4 pigs each. One group was fed basal ration A, another was fed a ration containing 25% alfalfa meal, and the third was fed one containing 25% oat hulls. These ingredients were substituted for corn meal in

basal ration A. The pigs were maintained on their respective rations for at least 10 days before receiving 150 μc of cesium-134 orally (Hansard et al., '51). Five days after administration of the isotope, the pigs were sacrificed and tissues removed for assay. The pigs had an average weight of 100 lbs. at time of sacrifice.

In the second experiment, 20 Hampshire pigs were fed basal ration B for a period of at least 7 days. Half the pigs were then fed a ration containing 20% beet pulp, substituted for corn starch, for a period of 7 days. One hundred microcuries of cesium-134 were administered intraperitoneally to half the pigs on each ration and 2 mc of potassium-42 to the remaining pigs. A 72-hour balance trial was obtained from those animals that received cesium-134 and a 48-hour balance trial from those that received potassium-42. The relatively short half life of potassium-42 (12.5 hours as compared to 2.3 years for cesium-134) necessitated the use of such a large dose and a shorter balance trial. Cesium-134 chloride was administered at tracer levels while the potassium-42 dosage contained 500 mg of potassium chloride. At the time of sacrifice, the pigs had an average weight of 43 lbs.

In the third experiment, 30 pigs of mixed ancestry with an average weight of 41 lbs. were divided into 5 groups of 6 pigs each. They were all fed basal ration C for one week. At the end of this period, one group was continued on basal ration C and the other fed basal ration C supplemented with either 0.2, 0.4, 0.6 or 0.8% potassium in the form of potassium chloride. After at least two weeks on their respective rations they were given 200 μc of cesium-134 intramuscularly. Five days after administration of the nuclide they were sacrificed and tissues removed for assay. The pigs had an average weight of 76 lbs. at time of sacrifice.

Excreta were collected at 24-hour intervals following the administration of the nuclides. Tissue (gastrocnemius muscle, kidney, liver, spleen, and heart) and urine samples were digested with hot nitric acid, and aliquots counted in a scintillation well-type counter. Duplicate samples of feces were

ashed at 600°C for 24 hours, dissolved in 1 ml of concentrated hydrochloric acid and 8 ml of concentrated nitric acid and aliquots counted. Feces were slightly contaminated with urine due to the method of collection in adapted commercial dog metabolism units. For ease of recording and reading, fecal, urinary and total excretory data have been reported utilizing a maximum of three significant figures and are not indicative of the sensitivity of the experiments.

RESULTS AND DISCUSSION

In the first experiment, table 2, there was observed a significant (5% level) decrease in cesium-134 uptake by the muscle, kidney, liver, spleen and heart tissues of pigs fed the ration containing alfalfa when compared to those fed the basal or oat hull ration. Significantly more cesium-134 was found in feces of pigs fed the alfalfa ration than in those fed the other two rations. The differences among the groups with regard to total excretion of cesium-134 were significant

TABLE 2

Influence of alfalfa and oat hulls on the excretory pattern and tissue retention of cesium-134

MEASUREMENT	DIET			L.S.D. [2]
	Basal A	Alfalfa [1]	Oat hulls [1]	
Tissues [3]				
Muscle	27	20	29	6
Kidney	44	27	40	10
Liver	21	15	21	3
Spleen	43	16	42	4
Heart	30	20	28	4
Total excretion [4]				
Urinary	15.0	16.6	7.7	5.5
Fecal	7.8	15.5	5.0	2.9
Total	22.8	32.1	12.7	7.5
Urinary/fecal ratio	1.9	1.1	1.5	

[1] Alfalfa or oat hulls replaced 25% of the corn meal of ration A.

[2] Least significant difference between means at the 5% level of confidence.

[3] Expressed as percentage of orally administered dose per gram of tissue $\times 10^4$.

[4] Expressed as percentage of orally administered dose.

with the greatest excretion occurring on the alfalfa ration and the least on the oat hull ration. The decrease in cesium-134 excretion by pigs fed the oat hull ration might be explained by the decrease in growth rate (7 lbs. less than the controls) experienced by pigs fed that ration which was marginal in both protein and fiber contents.

TABLE 3

Influence of beet pulp on the excretory pattern of cesium-134 and potassium-42

MEASUREMENT	CESIUM-134		POTASSIUM-42	
	Basal B	Beet pulp [1]	Basal B	Beet pulp [1]
Tissues [2]				
Plasma	3	3	7	4
Red blood cells	21	22	37	36
Muscle	85	66	89	85
Kidney	109	131	60	63
Liver	51	48	71	73
Spleen	63	64	93	91
Heart	83	82	89	73
Total excretion [3]				
Urinary	9.6	12.6	0.28	0.63
Fecal	5.4	12.6 [4]	0.98	2.52 [4]
Total	15.0	25.2 [4]	1.26	3.15 [4]
Urinary/fecal ratio	1.8	1.0	0.29	0.25

[1] Beet pulp replaced 20% of the cornstarch of ration B.

[2] Expressed as percentage of intraperitoneally administered dose per gram of tissue. $\times 10^4$.

[3] Expressed as percentage of intrapertoneally administered dose.

[4] Significant at the 1% level of confidence.

In the second experiment, table 3, no significant (5% level) differences due to treatment were observed in uptake of cesium-134 or potassium-42 by the tissues examined or in urinary excretion of these nuclides. Both fecal and total excretion of cesium-134 and potassium-42 were significantly (1% level) higher from pigs fed beet pulp than from those fed the basal ration.

The level of potassium fed in the third experiment, table 4, had no significant effect on the body weight gains. Signifi-

cantly more cesium-134 was found in the muscle tissue and less excreted by pigs that received no potassium supplementation than those receiving such supplementation. Significant increases in cesium-134 uptake by kidney and heart tissues were observed in pigs which received zero and 0.2% potassium supplementation when compared to those receiving 0.4% or more potassium. If one postulates that cesium-134 substitutes for the potassium in a deficient animal, the results may be interpreted to indicate that the requirement of the animal

TABLE 4

Influence of dietary potassium on the metabolism of cesium-134 in swine

MEASUREMENT	POTASSIUM ADDED TO RATION C (%)					L.S.D.[1]
	0.0	0.2	0.4	0.6	0.8	
Tissues [2]						
Muscle	47	34	32	31	36	9
Kidney	69	61	49	51	53	15
Liver	31	29	25	23	27	N.S.[3]
Spleen	30	31	25	24	25	6
Heart	42	37	29	30	31	7
Total excretion [4]						
Urinary	7.8	20.9	29.0	29.4	29.0	5.2
Fecal	1.6	4.2	4.8	5.5	5.2	2.3
Total	9.4	25.1	33.8	34.9	34.2	6.2

[1] Least significant difference between means at the 5% level of confidence.
[2] Expressed as percentage of orally administered dose per gram of tissue $\times 10^4$.
[3] Not significant.
[4] Expressed as percentage of orally administered dose.

lies between zero and 0.4% of potassium and probably below 0.2%. The National Research Council's ('53) recommendation for pigs of this size is 0.11% potassium. The fact that additional increments of potassium beyond 0.4% of potassium do not significantly increase cesium-134 excretion possibly may be explained by isotope dilution. Inasmuch as cesium-134 is present at tracer levels and potassium at considerably higher levels, and the tissues are saturated with potassium in those pigs receiving the higher levels of potassium, additional increments of potassium would have little influence on the equilibrium between potassium and cesium.

The significant increase in total excretion of cesium-134 and potassium-42 by pigs fed beet pulp and of cesium-134 by those fed alfalfa over those fed the basal ration was primarily due to the increase in the amount found in the feces (more than could be explained by chance contamination with urine). However, the significant differences in cesium-134 excretion in the experiment with different levels of potassium were primarily a function of the amount found in the urine. It would appear therefore that the mechanism explaining the increased cesium-134 excretion would not be the same in each instance. This theory is further strengthened by the observation that alfalfa and beet pulp analyze 2 and 0.18% potassium, respectively and yet express a similar influence on cesium-134 excretion. *In vitro* studies with both alfalfa and beet pulp (Mraz and Patrick, '57) show preferential adsorption of cesium-134 ions, even when they are present in tracer amounts in the presence of 0.1 N solutions of potassium, hydrogen, or sodium ions. It would appear therefore that the changes in excretory pattern of cesium-134 and potassium-42 are due to the alfalfa and beet pulp in the gut preferentially adsorbing the nuclides during their passage from the body fluids into the gut. If no materials are present in the gut to adsorb or remove these materials, most of the nuclides pass back into the body fluids and are either excreted through the kidneys or recycled through the body and eventually reach the gut again and the cycle continued.

SUMMARY

Sixty-two pigs were used in the three investigations reported. The first experiment tested the influence of alfalfa and oat hulls on the metabolism of orally administered cesium-134. the second, the influence of beet pulp on the metabolism of intraperitoneally administered cesium-134 and potassium-42, and the third the influence of dietary potassium on the metabolism of intramuscularly administered cesium-134.

It was observed that alfalfa and beet pulp significantly increased fecal and total excretion of cesium-134 and decreased

the ratios of urinary to fecal cesium-134. Beet pulp significantly increased fecal and total excretion of potassium-42 but did not appreciably influence the ratio. A deficiency in dietary potassium is marked by an increase in tissue uptake and a decrease in excretion of cesium-134.

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THE EFFECT OF RESTRICTED FOOD INTAKE ON THE LIFE SPAN OF GENETICALLY OBESE MICE [1]

PRISCILLA W. LANE AND MARGARET M. DICKIE
Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

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The effect of diet on the life span of experimental animals has been thoroughly reviewed by Silberberg and Silberberg ('55). Conspicuously absent from the literature is any study of the effect of food restriction on the life span of an experimental animal in which the obesity is hereditary. This report is presented to show the effect of long-term food restriction on the life span of genetically obese mice (Ingalls et al., '50).

EXPERIMENTAL

Three groups of mice, totaling 55 animals, were used in this experiment. All animals were obtained from the hereditary obese stock of mice of this laboratory. Group I, composed of 17 obese animals (genotype *obob*), was allowed food ad libitum for life. Group II, composed of 16 obese mice (genotype *obob*), was restricted in food intake from 6 weeks of age until death. Group III, composed of 22 thin siblings (genotype *ob*+ or ++) of the obese mice in group I and group II, was fed ad libitum. All three groups of mice were maintained on custom made mouse pellets.[2]

The animals were housed in solid bottom wooden cages, two to a cage and allowed water ad libitum. The restricted allow-

[1] This work has been supported by an institutional research grant from the American Cancer Society.

[2] Devised by Dr. H. P. Morris of the National Cancer Institute. The composition of this food is as follows: crude protein 17%, crude fat 7%, crude fibre 2% and nitrogen-free extract 55%. The ingredients of the food are: dried skim milk, ground wheat, brewers' yeast, corn oil, vitamin A feeding oil, D activated plant sterol, 1.4% salt and 0.13% iron citrate.

ance of food for group II was adjusted in order to maintain the weight of each animal as close to the mean weight of the thin siblings as was possible. All animals in group II were fed once daily except Sunday. Each animal was weighed bi-weekly from two months of age throughout life. All animals alive at 820 days of age were killed shortly thereafter, except for two obese females in group II which were fed ad libitum, starting at 800 days, for the remainder of their lives. Since all animals that survived to 820 days were either in group II or group III termination of the experiment did not affect the relationship between restricted food intake and longevity of obese mice.

RESULTS AND DISCUSSION

Figure 1 shows the mean body weight and range of each group at bimonthly ages. Obese mice fed ad libitum reached a maximum mean body weight of 83 gm at 14 months of age. The highest individual weight attained was 107 gm at 16 months. This same obese mouse also survived longer than any other in group I.

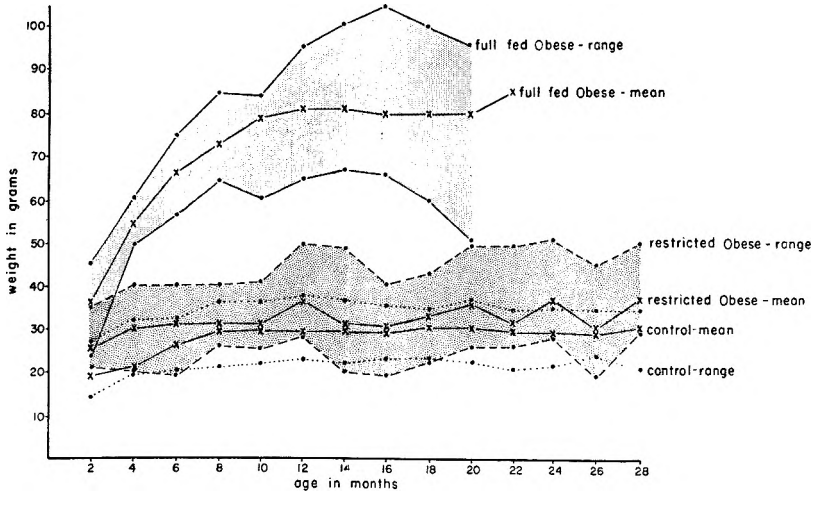

Fig. 1 Mean body weights and range of each group of mice at bimonthly ages.

The obese animals on restricted food intake reached a maximum mean weight of 36 gm at 12 months. Although some fluctuation occurred in the means of group II after 10 months of age, the means remained near the upper limit of the range in the control group. Fluctuations were probably due to the fact that two animals shared a cage and no attempt was made to control equal division of the daily ration within a cage. Obese mice on limited food intake appeared somewhat emaciated, particularly in the extremities, although they were maintained at weights (30 to 40 gm) similar to non-obese siblings. This observation is consistent with the report of Alonso and Maren ('55) that genetically obese mice on restricted food intake had more body fat but less body protein than did normal controls and that obese animals fed ad libitum exhibited considerably more fat than either non-obese controls or obese animals on limited food but normal body protein.

The maximum mean weight of the thin siblings was 30 gm. This was reached at 8 months of age and continued with little variation thereafter.

All animals in group I were continued on the experiment until death. However, the mean life span of the mice in both group II and group III was experimentally shortened since all animals still alive at 820 days (except for the two obese females in group II mentioned earlier) were killed. The mean life span of the obese animals fed ad libitum was 457 days, 290 days less than the mean of 747 days of the thin non-obese controls and 338 days less than the mean of 795 days of the obese mice on restricted food intake (table 1).

Table 1 was prepared to show the relation betweeen mortality rate and the maximum individual body weight attained. The animals that show the lowest mortality rate are those in the restricted obese group that attained a maximum weight between 41 and 50 gm. No animal in this group died prior to 730 days. The apparent optimum weight for longevity of obese mice as judged by the response of group II seems to be in a heavier range than for the non-obese mice but well below that weight attained by obese animals allowed food ad libitum.

TABLE 1

Relation between maximum body weight and mortality in three experimental groups of mice

EXPERIMENTAL GROUPS	MAXIMUM WEIGHT	N	MORTALITY RATE IN DAYS				RESIDUAL 730 –	LIFE SPAN	
			60 – 183	184 – 365	366 – 548	549 – 730		Mean	Range
	gm							*days*	*days*
I. Obese, ad libitum feeding	50 – 60	1		1					
	61 – 70	4	1	2		1			
	71 – 80	3			1	2			
	81 – 90	5		1	3	1			
	91 – 100	3			2	1			
	101 – 110	1				1			
	Total	17	1	4	6	6	0 (0%)	457	167 – 667
II. Obese, restricted feeding	30 – 40	7			1	2	4		
	41 – 50	8					8 [1]		
	51 – 60	1					1 [1]		
	Total	16			1	2	13 (81%)	795	547 – 1027
III. Nonobese	20 – 30	12				6	6		
	31 – 40	10		1	1	1	7		
	Total	22		1	1	7	13 (60%)	747	393 – 890

[1] One animal in this group was fed ad libitum at 800 days.

Table 1 also shows the mortality rate or percentage of animals in each experimental group which died between the ages of 167 to 183 days, 184 to 365 days, 366 to 548 days, 549 to 730 days and after 730 days. The mortality rate of 4 animals for group I found between 184 and 365 days is not equalled in the restricted obese group within the entire experimental period and not in the non-obese control group until 549 to 730 days of age. None of the obese mice on ad libitum food intake lived beyond 667 days while 81% of the obese mice on limited food and 60% of the non-obese siblings lived beyond 730 days.

At 800 days two obese females on limited food intake were fed ad libitum for life. These animals lived to 1013 and 1027 days and the highest weights attained were 59 and 49 gm respectively. They did not reach, at this age, a weight as high as any full-fed obese animal in group I.

These results leave little doubt that overweight, resulting from overeating, shortens life span even in those animals whose genetic constitution makes overeating an instinctive performance. By long term restriction of food intake the life span of genetically obese mice can be increased well beyond that of obese mice allowed food ad libitum.

SUMMARY

Three groups of animals were used to determine the effect of restricted food intake on the longevity of hereditary obese mice. Obese mice in group I were fed ad libitum for life. Obese mice in group II were on limited food intake from 6 weeks of age for life and the thin sibling controls of group III, were allowed unlimited food.

The maximum mean weight for animals in group I was 83 gm, for animals in group II, 36 gm and for group III, 30 gm.

The body weight associated with longer life span in genetically obese mice was in a range greater than that normally attained by non-obese controls but considerably less than that reached by obese mice allowed to eat ad libitum.

The mean life span for obese mice fed ad libitum was 457 days, for restricted obese mice, 795 days and for the controls,

747 days. One hundred percent of the full-fed obese mice were dead by 667 days while 81% of the obese mice on limited food intake and 60% of the non-obese controls were still alive at 730 days.

These results show that severe obesity resulting from overeating in animals whose genetic constitution favors overfeeding greatly shortens the life span of these animals.

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RESPONSE OF RATS TO DIETS HIGH IN METHIONINE AND RELATED COMPOUNDS [1]

H. P. COHEN, H. C. CHOITZ AND C. P. BERG
Department of Biochemistry, State University of Iowa, Iowa City

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INTRODUCTION

Excessive dietary methionine is known to retard growth in the rat (Kade and Sheperd, '48; Brown and Allison, '48; Wretlind, '49; Van Pilsum and Berg, '50; Hardin and Hove, '51) and to produce splenic hemosiderosis (Van Pilsum and Berg, '50). Since it seemed possible that these responses were in some way associated with the catabolism of methionine, various metabolites have since been tested to determine what capacities they might have to produce either or both effects. Certain compounds which contain labile methyl groups, or are able to react with them, were also tried. The results obtained seem to indicate that the growth-retarding effect is associated with the metabolism of the homocysteine moiety of methionine, and that the production of excess hemosiderin in the spleen may be linked with its role as a transmethylating agent.

EXPERIMENTAL

Substances studied as possible metabolites of methionine were homocystine, methionine sulfoxide, homoserine and α-amino butyric acid. Compounds tried because of their capacity

[1] The data presented in this paper are taken from dissertations submitted by Harold P. Cohen and Harold C. Choitz in partial fulfillment of requirements for degrees of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa. The work was aided by grants from the Dow Chemical Company.

to yield methyl groups for transfer were choline, betaine and dimethylthetin. Serine and cystine were tested for possible influence upon the production of cystine from methionine via cystathionine. Glycocyamine and glycine and arginine were employed because they are known to be involved, along with methionine, in the production of creatine. Ethanolamine was tested because it may undergo methylation to choline.

MATERIALS

The DL-methionine used for feeding was prepared by recrystallization of the pharmaceutical grade available from the Dow Chemical Company. The feed grade of DL-methionine was employed in the preparation of the DL-homocystine, by decomposition with 18 N sulfuric acid, essentially as directed by Butz and du Vigneaud ('32), and in the preparation of inactive methionine sulfoxide [2] according to the method of Toennies and Kolb ('39), as modified by Waelsch and others ('46); also subsequently, as directed by Lepp and Dunn ('55). Because of the heavy adsorption of the homocystine on the barium sulfate it was eluted with dilute hydrochloric acid and precipitated by adding sodium acetate. The DL-homoserine was prepared for us by Dr. H. C. White of the Dow Chemical Company. The DL-α-amino-*n*-butyric acid was purchased from the Eastman Kodak Company. The total nitrogen content of each of these products compared favorably with the theoretical value.

Choline chloride and betaine hydrochloride were purchased from the Nutritional Biochemicals Co. The dimethylthetin chloride was prepared from dimethyl sulfide and chloroacetic acid by the method of Maw ('53). Ethanolamine hydrochloride was synthesized as directed by Jones ('44).

The glycocyamine was purchased from the Hoffman-La-Roche Co., the L-arginine monohydrochloride from Merck and

[2] Since oxidation of DL-methionine to its sulfoxide renders the sulfur atom asymmetric, two racemic modifications are formed (Toennies and Kolb, '39). To conform with Rule 9 of the American Chemical Society rules on nomenclature of natural amino acids and related substances (Crane, '52), this compound is therefore designated inactive methionine sulfoxide.

Co., and the DL-serine and L-cystine from the Nutritional Biochemicals Company. The glycine was provided by the Dow Chemical Company.

Feeding Tests. Individually housed weanling male rats of the Sprague-Dawley strain were used throughout. Except in paired feeding tests, they were given food ad libitum. Water was always available. Weighings were made every 4 days, usually at the same hour.

In most of the experiments the basal diet contained 12% of vitamin-free casein [3] and 0.6% of DL-methionine. In several later tests 18% of vitamin-free casein and 0.2% of DL-methionine were used. The diet also contained sucrose 15.0, Cellu flour 2.0, salt mixture 4.0 (Jones and Foster, '42), corn oil 2.0, vitamin A and D concentrate [4] 0.08, inositol 0.1, choline chloride 0.2, liver extract [5] 0.4, and dextrin to bring the total to 100 gm. When supplements were incorporated in the diet, an equal weight of dextrin was omitted. To each kilogram of diet were added the following vitamins: thiamine hydrochloride 5, riboflavin 10, pyridoxine hydrochloride 5, nicotinic acid 5, calcium pantothenate 25, *p*-aminobenzoic acid 300, α-tocopherol acetate 25, and 2-methyl-1, 4-naphthoquinone 2 mg, and biotin 100, folic acid 100, and vitamin B_{12} 15 μg.

At the termination of the feeding tests, the animals were sacrificed and their spleens, and occasionally other tissues, were examined for the deposition of iron. In the earlier tests, this was done histologically by the conversion of the iron in the hemosiderin granules to Prussian blue. In the later tests, non-hemin iron was extracted from the homogenized tissue by heating it with equal volumes of 10% trichloracetic acid and 4% sodium pyrophosphate. This is a minor modification of the procedure of Brückmann and Zondek ('40). The iron was reduced to the ferrous state by buffering the extract and adding hydroquinone, then measured by estimating the color

[3] "Vitamin Test" casein, Nutritional Biochemicals Corporation.

[4] Oleum Percomorphum, Mead Johnson and Company. It contained 60,000 U.S.P. units of vitamin A and 8,500 U.S.P. units of vitamin D per gram.

[5] Liver concentrate N.F., made available through the generosity of Dr. S. W. Hier and the Wilson Laboratories.

development upon standing 24 hours after the addition of of 1,10-phenanthroline. This is essentially the procedure of Hummel and Willard ('38).

RESULTS AND DISCUSSION

The marked growth depression commonly observed when an excess of methionine approximating 1.8% is added to the diet is shown in table 1. In this instance the basal diet contained 12% of casein and 0.6% of methionine. When homocystine, equivalent in sulfur content, was provided as the supplement instead of methionine, it produced a growth depression which was possibly even more severe, but L-cystine, which may be derived from cysteine produced metabolically from homocysteine and serine (Binkley and du Vigneaud, '42; Stetten, '42) showed little, if any, growth-retarding capacity. Supplementation with both methionine and glycocyamine led to a depression in growth somewhat greater than with methionine alone. Glycocyamine is known to be synthesized in metabolism from glycine and arginine (Borsook and Dubnoff, '41; Bloch and Schoenheimer, '41) and to undergo methylation to creatine in the presence of methionine (Borsook and Dubnoff, '40). Curiously, further supplementation of the high methionine diet with arginine and glycine, instead of glycocyamine, alleviated the growth depression. Our preliminary report of this observation (Cohen and Berg, '51) has been verified by Hardin and Hove ('51). It is obvious that the provision of arginine and glycine could be expected to produce the same response only if their conversion into glycocyamine were an obligate step. Brown and Allison ('48) found that the addition of both arginine and methionine to the diet increased the excretion of creatinine more than did the addition of either alone. Hardin and Hove ('51) observed a considerably greater urinary creatine output upon adding glycocyamine to their high methionine diets than upon adding arginine and glycine. Supplementation of our high methionine diet with DL-serine alleviated the growth depression somewhat, but

TABLE 1

Growth response in rats fed excess methionine with or without various other dietary supplements for 28 days

GROUP [1]	EXCESS METHIONINE OR DERIVATIVE [2]	OTHER SUPPLEMENT	AVERAGE GAIN IN WEIGHT	
			Per day	Per gm of food
			gm	*gm*
Series I				
1	None	None	5.03 ± 0.24 [3]	0.40
2	1.8% DL-Methionine	None	2.19 ± 0.14	0.35
3	1.8% DL-Methionine	1.41% Glycocyamine	1.76 ± 0.12	0.31
4	1.8% DL-Methionine	0.91% Glycine + 2.12% arginine	3.00 ± 0.12	0.40
5	1.8% DL-Methionine	1.27% DL-Serine	2.42 ± 0.10	0.36
6	1.62% DL-Homocystine	None	1.95 ± 0.11	0.31
Series II				
7	None	None	5.13 ± 0.18	0.38
8	1.8% DL-Methionine	None	2.21 ± 0.17	0.31
9	None	1.45% L-Cystine	4.80 ± 0.29	0.39
10	1.8% DL-Methionine	0.6% Choline chloride	2.06 ± 0.09	0.32
11	None	0.6% Choline chloride	5.19 ± 0.19	0.38

[1] In series I, group 6 consisted of 4, all other groups of 6 rats each; in series II each group consisted of 5 rats.
[2] The basal diet used in these series contained 12% of casein and 0.6% of DL-methionine.
[3] Standard error of the mean.

supplementation with 0.6% of chloine chloride may have aggravated it slightly.

Table 2 shows that relatively large additions (1.8%) of choline chloride or betaine hydrochloride to the basal diet did not in themselves markedly depress growth; when added to the diets supplemented with homocystine they alleviated the growth depression, though somewhat less effectively than did approximately twice the equivalent level of glycine. Known metabolic interrelations (Stetten, '41) indicate that choline and betaine may not only undergo transmethylation, but also conversion to glycine. At equivalent levels, serine accerlated the growth on homocystine, but cystine retarded it. These are responses which could possibly reflect influences upon the conversion of homocysteine to cysteine via cystathionine. Similarity of the growth acceleration observed when ethanolamine was added to the high methionine diet to that noted when the homocystine diet was supplemented with choline is consistent with the possible conversion of ethanolamine and methionine into choline and homocysteine, or vice versa, by transmethylation. Conversion of ethanclamine to glycine is another possibility.

Supplementation of the basal diet with methionine sulfoxide, which is able to replace methionine for growth (Bennett, '39), produced little growth retardation. Nor was any retardation effected by α-amino-*n*-butyric acid, which has been found in the plasma and urine of normal human subjects fed methionine, presumably as a consequence of its direct desulfurization (Dent, '47). Homoserine, which may be formed in the conversion of cystathionine to cysteine (Anslow and du Vigneaud, '47), also showed no tendency to retard growth on the basal diet, but many even have stimulated it.

Histological examination of tissue sections from the rats whose growth is recorded in tables 1 and 2 confirmed the observation of Van Pilsum and Berg ('51) that diets high in methionine also promote deposition of hemosiderin in the spleen, but apparently not in other tissues. Homocystine failed to produce this response. When it was fed along with

TABLE 2

Growth response in rats fed excess methionine with or without various other dietary supplements for 52 days

GROUP [1]	EXCESS METHIONINE OR DERIVATIVE [2]	OTHER SUPPLEMENT	AVERAGE GAIN IN WEIGHT	
			Per day	Per gm of food
			gm	*gm*
Series III				
1	None	None	4.45 ± 0.11 [3]	0.33
2	1.8% DL-Methionine	None	2.37 ± 0.18	0.27
3	1.62% DL-Homocystine	None	2.09 ± 0.20	0.29
4	1.62% DL-Homocystine	1.8% Choline chloride	2.96 ± 0.13	0.34
5	1.62% DL-Homocystine	1.8% Betaine hydrochloride	3.37 ± 0.16	0.32
6	None	1.8% Choline chloride	4.00 ± 0.19	0.33
7	None	1.8% Betaine hydrochloride	4.37 ± 0.11	0.34
8	1.62% DL-Homocystine	1.82% Glycine	3.88 ± 0.36	0.35
9	1.8% DL-Methionine	1.82% Glycine	3.44 ± 0.17	0.34
10	1.62% DL-Homocystine	1.27% DL-Serine	3.01 ± 0.26	0.31
11	1.62% DL-Homocystine	1.45% L-Cystine	1.64 ± 0.32	0.24
12	1.44% DL-Homoserine	None	5.09 ± 0.07	0.32
Series IV				
13	None	None	5.17 ± 0.06	0.32
14	1.8% DL-Methionine	None	2.71 ± 0.01	0.29
15	None	1.8% Choline chloride	4.48 ± 0.15	0.32
16	None	1.18% Ethanolamine hydrochloride	4.86 ± 0.11	0.32
17	1.8% DL-Methionine	1.18% Ethanolamine hydrochloride	3.37 ± 0.16	0.32
18	1.98% Inactive methionine sulfoxide	None	4.72 ± 0.15	0.31
19	1.24% DL-α-amino-*n*-butyric acid	None	4.93 ± 0.11	0.31

[1] In series II, group 1 contained 8 animals, group 2 contained 5, all others 6 each. In series IV, group 13 contained 40 animals, group 14 contained 25, group 16 contained 4, all others contained 5 each.

[2] The basal diet in these series contained 12% of casein and 0.6% of DL-methionine.

[3] Standard error of the mean.

choline or betaine, the incidence of hemosiderosis was no greater than when either of the latter was fed alone. The spleens of rats fed glycine, serine or cystine, plus homocystine, were normal. In the rats fed basal diets supplemented with 0.6% of extra choline, the granules of hemosiderin seemed to exceed the number found in the spleens of rats fed the basal diet. At the 1.8% level, choline produced slightly greater hemosiderin deposition than did betaine.

Further supplementation of the high-methionine diets with glycocyamine, with glycine and arginine, or with serine did not seem to diminish the hemosiderin deposition promoted characteristically by diets high in methionine alone.

The spleens of animals fed homoserine and α-aminobutyric acid were normal. Those of rats fed supplements of methionine sulfoxide showed marked deposition of hemosiderin, though somewhat less than in the spleens of rats fed excess methionine.

Table 3 summarizes the results obtained in a later study in which the basal diet contained 18% of casein and 0.2% of DL-methionine. The rate of growth was greater than on the 12% casein-0.6% DL-methionine diet, and the growth depression induced by supplementation with DL-methionine exceeded that induced by DL-homocystine. This may be attributable to the greater surplus of exogenous amino acids provided by the 18% casein diet. On the 12% casein diet addition of glycine alleviated the growth depression induced by homocystine more readily than it did that induced by methionine. Choline chloride and betaine hydrochloride produced only minor growth retardation, methione sulfoxide somewhat more. Dimethylthetin chloride was the severest growth retardant. It was employed because it is known to be an extremely effective methyl donor, at least for transmethylation with homocysteine (du Vigneaud, Moyer and Chandler, '48; Dubnoff and Borsook, '48). It induced deposition of non-hemin splenic iron equal to that noted with methionine. In this capacity, homocystine was the least effective agent tested and betaine hydro-

TABLE 3

The influence in rats of feeding excess methionine or other dietary supplements for 36 days on growth, erythrocyte count and splenic iron

GROUP [1]	DIETARY SUPPLEMENT [2]	AVERAGE GAIN IN WEIGHT		AVERAGE ERYTHROCYTE COUNT	Fe/100 GM OF WET SPLENIC TISSUE
		Per day	Per gm of food		
		gm	*gm*	*per mm³ × 10⁻⁶*	*mg*
Series V					
1	None	6.2 ± 0.10 [3]	0.40	8.86	92 ± 11 [3]
2	1.8% DL-Methionine	3.2 ± 0.22	0.37	7.43	249 ± 23
3	1.85% Betaine hydrochloride	5.6 ± 0.24	0.40	8.59	122 ± 11
4	1.68% Choline chloride	5.8 ± 0.06	0.42	9.00	129 ± 9
5	1.89% Dimethylthetin chloride	2.0 ± 0.08	0.32	7.96	272 ± 22
6	1.62% DL-Homocystine	3.8 ± 0.25	0.38	8.22	114 ± 14
7	1.98% Inactive methionine sulfoxide	4.9 ± 0.15	0.39	8.18	125 ± 10
8	1.8% DL-Methionine plus additional vitamins	3.1 ± 0.14	0.35	7.11	316 ± 17
Series VI [4]					
9	None	6.1 ± 0.11	0.43	8.64	
10	1.8% DL-Methionine	3.7 ± 0.19	0.41	6.94	
11	None	3.9 ± 0.16	0.42	8.90	

[1] Groups 1 and 3 consisted of 4 rats each, groups 2 and 4 to 8 of 5, and groups 9 to 11 of 8 rats each.

[2] The unsupplemented basal diet in series V and VI contained 18% of casein and 0.2% of DL-methionine.

[3] Standard error of the mean.

[4] In series VI groups 9 and 10 were fed ad libitum; the dietary intake per day of each of the animals in group 11 was restricted to the average daily intake of the animals in group 10.

chloride, methionine sulfoxide and choline chloride were mildly stimulatory.

The effect of supplementing a high-methionine diet with extra vitamins has been tested repeatedly, because it seemed possible that a vitamin deficiency could have been precipitated by reactions concerned with the catabolism of the excess methionine. In the representative comparison presented (series V, table 3, group 8 vs. group 2) the following extra vitamins were added per kilogram to the diet: riboflavin 90, pyridoxine hydrochloride 45, nicotinic acid 45, calcium pantothenate 225, 2-methyl-1, 4-naphthoquinone 18, and folic acid 1.9 mg, vitamin B_{12} 135 μg, and inositol 1 gm. In none of the several tests, of which this is representative, was any protective or alleviating effect noted. On the contrary, erythrocyte turnover was apparently accelerated. It is conceivable that transmethylation may have been stimulated.

The possibility that the lowered food consumption could have caused the characteristic responses to the high-methionine diets was checked by restricting the daily intake of the rats fed the basal diet to the average daily intake of the animals fed the high-methionine diet (series VI, table 3). Weight gains on the restricted regimen were only slightly higher, but the erythrocyte counts were normal. In no instance did the spleens of the rats fed the restricted basal diet show the hypertrophy and darkening induced by the high-methionine diet. Unfortunately an unanticipated mishap prevented proper completion of the analyses of the spleens for non-hemin iron.

The evidence presented is, of course, only circumstantial, but it does seem to indicate that the depression of growth induced by diets containing excess methionine may be associated with the metabolism of the homocysteine moiety; and the increased deposition of hemosiderin with the labilization or transfer of its methyl group. On the basis of evidence that methionine may completely inhibit the active transport of other single monoamino-monocarboxylic amino acids through everted sacs of the intestine (Wiseman, '55), Ghadially and Wiseman ('56) have assumed that methionine "probably

produces its growth inhibiting effect by interfering with the active uptake of essential amino acids by the normal tissues of the animal, and hence interferes with protein synthesis." This assumption is not consistent with the observation of Cohen and Berg ('56) that diets high in methionine accelerate erythrocyte turnover, hence undoubtedly also the synthesis of the protein fraction of hemoglobin.

Evidence for the possible relationship of hemosiderin deposition to transmethylation is less convincing than desirable, but perhaps close correlation between the various agents employed can hardly be expected. Dubnoff and Borsook ('48) have emphasized the probability that there are "specific methyl donors for each methyl acceptor"—"and that a given methyl compound may be related to another only indirectly through a series of methyl transfer reactions," also that some transmethylation reactions occur only under aerobic conditions, whereas others may take place anaerobically. Of all of the methyl donors tested, dimethylthetin was the only one found whose capacity to lower the erythrocyte count or promote the deposition of non-hemin splenic iron approximated quantitatively that of DL-methionine.

In tests with liver slices, Handler and Bernheim ('43) observed that methionine sulfoxide did not appreciably enhance the methylation of glycocyamine. Borsook and Dubnoff ('47) found that it contributed more slowly to creatine formation than did methionine. In the intact animal (rat), on the other hand, Stekol ('55) observed that intraperitoneally injected methionine sulfoxide-CH_3-C^{14} was converted to methionine, choline and creatine. The rate of creatine formation recorded was superior to that observed with methionine-CH_3-C^{14}. In mice fed low protein-high fat diets, methionine sulfoxide was an effective lipotropic agent (Singal and Eckstein, '41). The evidence afforded by these data and by our own, particularly on the 12% casein diet, is suggestive, but too circumstantial to warrant concluding definitely that methionine sulfoxide transfers its methyl group directly, rather than indirectly through slow conversion to methionine.

Since the course and rates of metabolism of enantiomorphs often differ, use of the L-isomers instead of the racemates employed in this study may be expected to yield results quantitatively divergent from those recorded here. Chances for such deviation would probably be greatest in the case of methionine sulfoxide, which contains two differing asymmetric centers (Toennies and Kolb, '39; Waelsch and others, '46).

SUMMARY

The growth retardation and accelerated deposition of splenic iron noted upon adding excess methionine to a basal diet were not markedly altered by the further addition of glycocyamine to favor creatine and homocysteine production. Supplementation with glycine, with glycine and arginine, or with ethanolamine decreased the growth-retarding effect of the excess methione, but apparently not its stimulation of iron deposition.

Replacement of the excess methionine by homocystine produced the same striking retardation of growth, but little or no increase in splenic iron. Methionine sulfoxide produced less growth retardation than homocystine, but was more effective in stimulating the deposition of splenic iron.

Supplementation of the basal diet with cystine, α-amino-*n*-butyric acid, or homoserine produced little change in the rate of growth and no stimulation of iron deposition. Cystine and homocystine together suppressed the rate of growth more markedly than homocystine alone.

Dimethylthetin was as effective as methionine in suppressing growth and stimulating the deposition of splenic iron. Choline and betaine alone produced moderate increments in splenic iron, but little or no growth retardation. When they were fed in diets which contained homocystine, they decreased the growth depression as effectively as glycine or serine, presumably because they may undergo conversion to glycine, but feeding them jointly with homocystine did not alter markedly their capacities to promote the deposition of splenic iron.

The provision of extra vitamins did not alleviate the growth suppression or the iron deposition induced by excess methionine. Paired feeding showed that the limited intake of the high methionine diets was probably largely responsible for the growth retardation, but not for the characteristic decrease in the erythrocyte count.

It is tentatively assumed that the accelerating effect of methionine upon the erythrocyte turnover is associated primarily with its participation in transmethylation reactions, its growth-depressing effect primarily with the metabolism of its homocysteine moiety.

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CAROTENE UTILIZATION AND CHOLESTEROL METABOLISM AS INFLUENCED BY ADDED CHOLINE AND VITAMIN B_{12} TO DIETS CONTAINING YEAST OR A SYNTHETIC VITAMIN MIXTURE [1]

HELEN L. MAYFIELD AND RICHARD R. ROEHM
Department of Home Economics Research, Montana State College Agricultural Experiment Station, Bozeman

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Reports indicate that choline, vitamin B_{12}, methionine and folic acid have lipotropic properties. Furthermore, these substances may be interrelated in their combined effect on methylation reactions as well as in other biological processes (Drill, '54; McCormick and Drill, '50; Strength, Schaefer and Salmon, '51; Schaefer, Salmon, Strength and Copeland, '50; Hawk and Elvehjem, '53; and Best, Lucas, Patterson and Ridout, '53). A relationship between serum cholesterol and the presence of choline in the diet was found by Ridout, Patterson, Lucas and Best ('54). Other reports have shown that certain of these same lipotropic factors may influence the rat's utilization of carotene (Mayfield and Roehm, '56; Popper and Chinn, '42; Clayton and Baumann, '44; High and Wilson, '53). The authors in a previous study (Mayfield and Roehm, '56) found that rats fed vitamin B_{12} with a yeast-containing diet

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had greater utilization of carotene than did those receiving vitamin B_{12} with a synthetic-vitamin-mix diet, indicating the possible presence of some influencing factors (or factor) in brewers' yeast. McCormick and Drill ('50) and Drill ('54) have reported that brewers' yeast, under certain circumstances, exerted a greater lipotropic effect than did their combinations of choline, vitamin B_{12} and folic acid.

This paper reports the effects of dietary choline and vitamin B_{12} on the utilization of carotene by the rat and the accompanying serum and liver cholesterol values when the rats received either a synthetic-vitamin-mixture diet or a yeast-containing diet.

EXPERIMENTAL PROCEDURE

Weanling rats [2] three weeks old and weighing 40 to 50 gm were assigned at random to 10 experimental groups each containing 5 females and 5 males. The rats were placed in individual metal cages with raised screen bottoms and fed the experimental diets ad libitum. Weight records and food consumptions were recorded during both the depletion and test periods.

Rats in groups 1 through 7 received diet I, vitamin A-free, and containing a synthetic vitamin mixture as the source of the B vitamins. Diet 1 consisted of: vitamin A-free test casein,[3] 18%; cornstarch, 60%; cottonseed oil,[4] 13%; salt mixture,[5] 4%; and vitamin mixtures,[6] 5%. This vitamin mixture differed from that used in previous work (Mayfield and Roehm, '56) in that choline which had been added at the rate of 250 mg per 100 gm of diet was now added in amounts vary-

[2] Obtained from Holtzman Rat Company, Madison, Wisconsin.

[3] "Vitamin Test" casein, Nutritional Bioehemicals Corporation, Cleveland, Ohio.

[4] Wesson Oil.

[5] Salt Mixture, U.S.P. XIV, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[6] The vitamin mixture was prepared as follows: pyridoxine hydrochloride 0.63 mg, calcium pantothenate 5.0 mg, niacin 0.63 mg, *p*-aminobenzoic acid 30.0 mg, inositol 100 mg, vitamin K 0.2 mg, biotin (free acid) 10 μg, riboflavin 0.4 mg, thiamine hydrochloride 0.5 mg and cornstarch to make 5.0 gm.

ing from 30 to 350 mg. One group of controls receiving no choline during the test period was also included. Folic acid which had previously been added at the rate of 200 μg per 100 gm of diet was omitted from the present mixture. The 30 mg level of choline was chosen since that represented the amount of choline supplied by the yeast [7] in diet 2. The largest level of choline fed, 350 mg per 100 gm of diet has been reported as adequate for growth, reproduction and lactation in the rat (Knudson and Harris, '55; MacLean, Ridout and Best, '37).

During the vitamin A depletion period the diet was supplemented with 30 mg of choline per 100 gm of diet. Four days before the rats were expected to be be depleted of vitamin A the choline supplement was discontinued. Rats in groups 8, 9 and 10 received diet 2, vitamin A-free, and containing yeast as the source of the B-vitamins. This diet consisted of: vitamin A-free test casein, 18%; cornstarch, 57%; cottonseed oil, 13%; salt mixture 4%; and brewers' yeast,[8] 8%. This diet was fed without supplements during the vitamin A depletion period. Both diets 1 and 2 were provided with vitamin D at the level of 3 U.S.P. units per gram of diet by the addition of viosterol [9] to the cottonseed oil.

All rats were fed these vitamin A-free test diets until they showed evidence of vitamin A deficiency as characterized by declining weight or ophthalmia, or both. At the beginning of the experimental period each rat was fed daily a supplement of 60 μg of carotene [10] dissolved in 0.2 ml of cottonseed oil containing 0.50 mg of added alpha-tocopherol. The carotene supplement was pipetted directly into the mouths of the rats. Rats in group 1 received no supplement of choline; those in

[7] Choline analysis of yeast through courtesy of Dr. Karl F. Swingle, Veterinary Research Laboratory, Montana State College.

[8] Brewers' Yeast Powder, General Biochemicals, Inc., Chagrin Falls, Ohio.

[9] Viosterol, irradiated ergosterol, 400,000 U.S.P. units of vitamin D per gm, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[10] Carotene, 90% beta and 10% alpha, obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

group 2 received 30 mg choline; group 3, 60 mg; group 4, 100 mg; group 5, 250 mg; group 6, 350 mg; group 7, 250 mg choline and 300 mμg vitamin B_{12}[11] (Mayfield and Roehm, '56) per 100 gm of diet. Rats in group 8 received no supplement of choline but received 30 mg of choline per 100 gm of diet supplied by the yeast; group 9 received additional choline to equal 350 mg; group 10 received no additional choline but was supplemented with 3000 mμg vitamin B_{12} per 100 gm of diet.

The supplements were fed for 14 days. Twenty-four hours after the last carotene supplement was fed, the rats were killed by decapitation and the blood collected for serum preparation. Kidneys and whole livers were removed, freed of foreign tissue, weighed and packaged in polyethelene wrap. The livers and serum were then frozen and held at −23°C until analyzed.

Vitamin A and cholesterol analyses. The livers and kidneys were analyzed for vitamin A following the method of Ames, Risley and Harris ('54) with minor modifications. The extract from this procedure was also used for the determination of cholesterol. Total serum cholesterol and cholesterol in the livers were determined by the method of Abell, Levy, Brodie and Kendall ('52). The Evelyn colorimeter was used for the vitamin A determinations and the Beckman B spectrophotometer for the cholesterol. The amount of vitamin A present in the liver and kidneys and the liver and serum cholesterol were calculated for each rat, and the means, together with the standard errors of the mean, were determined separately for males and females. The resulting data were treated statistically according to the analysis of variance (Ostle, '54).

RESULTS AND DISCUSSION

The levels of choline and vitamin B_{12} fed to the experimental groups, the estimated amounts of choline, vitamin B_{12} and folic acid furnished by the yeast in diet 2 together with the resulting vitamin A and cholesterol data are shown in table 1.

[11] Vitamin B_{12}, 0.1% Trituration (with mannitol), Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 1

Influence of adding choline and vitamin B_{12} to diets containing yeast or a synthetic-vitamin mixture on vitamin A storage and serum and liver cholesterol of rats fed 60 μg of carotene daily

GROUP NO.[1]	SUPPLEMENTS PER 100 GM DIET			GAIN IN BODY WEIGHT	WEIGHT OF LIVER		VITAMIN A IN		TOTAL CHOLESTEROL	
	Choline	Vitamin B_{12}	Folic acid		Total	Per 100 gm rat	Liver	Liver plus kidneys	Serum	Liver
	mg	*mμg*	*μg*	*gm*	*gm*	*gm*	*μg*	*μg*	*mg %*	*% moist wt.*
					Diet 1, containing synthetic-vitamin mixture[2]					
	Females									
1	0	0	0	30	6.1	4.9	119	124 ± 8.8[3]	102 ± 5.4	0.45 ± 0.02
2	30	0	0	33	6.1	4.7	127	131 ± 10.5	98 ± 3.2	0.38 ± 0.06
3	60	0	0	37	6.0	4.3	132	136 ± 12.0	110 ± 4.7	0.39 ± 0.03
4	100	0	0	38	5.9	4.3	128	132 ± 5.5	122 ± 5.9	0.39 ± 0.03
5	250	0	0	39	5.9	4.1	123	127 ± 5.4	120 ± 2.3	0.38 ± 0.02
6	350	0	0	43	6.0	4.2	115	120 ± 9.9	127 ± 7.3	0.34 ± 0.01
7	250	3000	0	41	5.6	3.7	145	148 ± 19.4	98 ± 12.0	0.38 ± 0.01
	Males									
1	0	0	0	45	7.5	5.3	72	86 ± 5.8	89 ± 6.8	0.45 ± 0.06
2	30	0	0	54	8.1	4.7	77	89 ± 12.5	105 ± 6.8	0.40 ± 0.05
3	60	0	0	52	7.0	4.6	64	76 ± 8.6	90 ± 5.2	0.36 ± 0.02
4	100	0	0	57	7.2	4.4	69	85 ± 13.8	96 ± 5.8	0.35 ± 0.03
5	250	0	0	61	7.2	4.3	75	87 ± 6.7	116 ± 6.7	0.33 ± 0.02
6	350	0	0	65	7.4	4.5	75	87 ± 8.1	123 ± 6.8	0.33 ± 0.02
7	250	3000	0	66	7.6	4.2	74	84 ± 8.6	116 ± 5.0	0.37 ± 0.06
					Diet 2, containing yeast[2]					
	Females									
8	30[4]	6[4]	176[4]	33	5.8	4.7	113	118 ± 14.1	91 ± 7.7	0.36 ± 0.03
9	350	6	176	30	5.3	4.3	139	142 ± 10.2	112 ± 4.5	0.37 ± 0.04
10	30	3006	176	35	6.0	4.1	185	188 ± 6.1	106 ± 8.0	0.35 ± 0.02
	Males									
8	30	6	176	44	7.6	5.3	72	83 ± 7.9	76 ± 4.4	0.31 ± 0.02
9	350	6	176	56	7.1	4.5	127	133 ± 7.8	127 ± 7.9	0.31 ± 0.02
10	30	3006	176	81	8.2	4.0	106	123 ± 11.2	98 ± 5.7	0.37 ± 0.01

[1] Ten rats per group, 5 females, 5 males.

[2] Synthetic-vitamin mixture and yeast described in experimental procedure.

[3] Standard error of the mean.

[4] These amounts of choline, vitamin B_{12} and folic acid furnished by yeast.

Because of the differences in response, the resulting data from males and females are presented separately. Rats in all groups showed signs of vitamin A deficiency in approximately 20 days. At this time they were placed on the experimental diets; they were about 6 weeks old and the females weighed from 92 to 113 and the males from 98 to 125 gm. The weights of the males are similar to the weights of certain groups of male rats fed choline-free diets for 35 to 42 days by Buckley and Hartroft ('54) in their study on the effects of choline on cardiovascular lesions. It is known that the very young rat will live only a short time without dietary choline or its precursors.

During the 14-day test period the mean gains in body weight of the 10 groups of females ranged from 30 to 43 gm and those of the males from 44 to 81 gm. Both male and female rats receiving the choline-free diet, group 1, continued to gain during the 14-day period. The mean weights of the livers from the female rats ranged from 5.3 to 6.1 gm and the body weights from 126 to 152 gm; in the male rats liver weights ranged from 7.0 to 8.2 gm and body weights from 140 to 204 gm. Liver weights per 100 gm of rat weight were calculated and together with the total liver weights are shown in table 1. Liver weights per 100 gm of rat weight for the various groups indicate that the rats receiving dietary choline had slightly smaller livers than did those on the choline-free diet, when expressed on this basis. Male and female rats in groups 7 and 10 receiving vitamin B_{12}, as well as choline, had somewhat smaller livers per 100 gm of rat than did those receiving only the choline.

Carotene utilization

Effect of added choline and vitamin B_{12} in synthetic vitamin-mix diets. Female rats. The average amounts of vitamin A in the whole livers plus kidneys of the various groups were treated according to the analysis of variance.[12] Feeding the various choline supplements, 30, 60, 100, 250 and 350 mg per

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

100 gm of diet (groups 2, 3, 4, 5 and 6) or feeding the choline-free diet (group 1) did not significantly change the female rat's ability to utilize carotene and the resulting storage of vitamin A. Rats in group 7 fed 3000 mμg of vitamin B_{12} per 100 gm of diet appeared to have a larger storage of vitamin A (148 μg) than did those in group 5 (127 μg), receiving no vitamin B_{12} and the same amount of choline; however, by statistical analysis the "F" value was 1.87 and the increase was not significant (F value of 4.04 necessary for significance at 0.05 level of probability). Since there was no significant difference in the vitamin A storage in the first 6 groups of rats receiving the various levels of choline, these groups, 1 through 6, were compared with group 7 receiving vitamin B_{12}. Again the difference was not significant with an "F" value of 3.43 and an "F" value of 4.04 necessary for significance at 0.05. Hence, feeding vitamin B_{12} to the female rats receiving the synthetic vitamin mixture, while showing a trend, did not alter significantly their ability to utilize carotene.

Male rats. Vitamin A storage in male rats was not altered by feeding the various levels of choline, from 30 to 350 mg of choline per 100 gm of synthetic-vitamin-mix diet, groups 2 through 6, or by feeding the choline-free diet, group 1. Neither did the addition of vitamin B_{12}, together with 250 mg of choline cause any change in carotene utilization when the synthetic vitamin-mix diet was fed. This negative response with the male rats is similar to that obtained with the female rats.

Effect of added choline and vitamin B_{12} in yeast containing diets. Female rats. When the female rats in group 8 were given the unsupplemented yeast diet, calculated to contain 30 mg of choline, 6 mμg of vitamin B_{12} and 176 μg of folic acid per 100 gm of diet their vitamin A storage (118 μg) was not significantly different from those in group 2 (131 μg) receiving the diet containing the synthetic vitamin mixture with 30 mg of choline and no vitamin B_{12} or folic acid. Rats in group 9 receiving the yeast diet with additional choline amounting to

350 mg appeared to have greater storage of vitamin A (142 μg) than those in group 6 (120 μg) receiving the same amount of choline and the synthetic-vitamin-mix diet; however, statistical analysis indicated that this increase was not significant with an "F" value of 2.04 (F value of 4.04 necessary for significance at 0.05). Likewise, rats in groups 9 receiving 350 mg of choline in the yeast-containing diet, appeared to store more vitamin A, (142 μg,) than did those in group 8 receiving 30 mg of choline (118 μg) but this increased storage was not statistically significant.

The addition of vitamin B_{12} to the yeast-containing diet, group 10, caused a significant increase in vitamin A storage (188 μg) as compared to 118 μg when the rats received the same diet with no vitamin B_{12} supplement, group 8, ($P < 0.01$).

Male rats. Feeding the unsupplemented yeast diet to the male rats in group 8 did not alter the storage of vitamin A from that obtained when the synthetic-vitamin-mix diet with the same amount of choline (30 mg) was fed to the male rats in group 2. This indicates that neither folic acid nor any other factor (or factors) which might be present in yeast had, by themselves, the ability to cause increased carotene utilization.

When the yeast-containing diet was supplemented with choline to equal 350 mg per 100 gm of diet (group 9) the male rats stored significantly more ($P < 0.01$) vitamin A (133 μg) than when the same diet was unsupplemented with choline, group 8 (83 μg) or when the same amount of choline (350 mg) was added to the synthetic-vitamin-mix diet, group 6 (87 μg) ($P < 0.01$). Thus the males responded in a positive and statistically significant manner to the 350 mg level of choline while the females did not respond to that extent. This increased storage may suggest that when yeast was present in the diet, the 350 mg level of choline served as a partial substitute for vitamin B_{12}.

The addition of 3000 mμg of vitamin B_{12} to the otherwise unsupplemented yeast diet, group 10, also caused a significant increase ($P < 0.01$) in vitamin A storage, 123 μg as compared

to 83 μg in group 8. This positive response to vitamin B_{12} by the male rats receiving the yeast-containing diet, but not by male rats receiving the synthetic-vitamin-mix diet, is similar to the results obtained with the female rats.

DISCUSSION

The results of this study, indicating that dietary choline did not affect the conversion of carotene to vitamin A as measured by liver and kidney storage, are in agreement with the findings of Clayton and Baumann ('44) who reported a normal distribution of vitamin A in the tissues of young rats with severe symptoms of choline deficiency. Popper and Chinn ('42) have reported that choline deficiency impaired the utilization of carotene and that the kidneys of rats on choline-poor diets were extremely rich in vitamin A. The distribution of vitamin A between the kidneys and liver of the rats used in the present study were similar regardless of their choline intake.

Previous work in this laboratory (Mayfield and Roehm, '56) indicated a similar response to the inclusion of vitamin B_{12} in the diet, namely, increased vitamin A storage when the diets contained yeast and no response when a somewhat different synthetic vitamin mixture was used as the source of the B vitamins. The previous vitamin mixture contained 200 μg of folic acid and a constant amount of choline, 250 mg, and the one used in this study had varying amounts of choline and no folic acid. While no folic acid was included in the synthetic vitamin mixture used in the present study it was included in the work previously reported (Mayfield and Roehm, '56) and similar results were obtained. Since folic acid was not included in the present study the possibility that folic acid may have been a contributing factor cannot be ruled out. However, these comparisons together with the comparison of groups 8 and 9 with groups 2 and 6 suggest that neither folic acid nor choline was the contributing factor in the response to the feeding of vitamin B_{12}. Both the yeast

and the synthetic-vitamin-mixture diets contained 18% of casein. However, the yeast diet contained about 4% of additional yeast protein which may have influenced the utilization of carotene, for it is known that both the quantity and balance of amino acids in the diet affect many physiological reactions. James and ElGindi ('53) found a relationship between carotene utilization and the type of protein fed. The present results indicating a positive effect of vitamin B_{12} on carotene conversion when the yeast containing diet was fed, as compared to a negative response when the synthetic-vitamin-mixture diet was fed, could also indicate the presence of some other factor (or factors) in the yeast diet which in conjunction with vitamin B_{12}, but not alone, had the ability to increase the conversion of carotene to vitamin A in these rats.

McCormick and Drill ('50) and Drill ('54) have reported on the lipotropic effects of liver extract, hog stomach, vitamin B_{12} concentrate, crystalline vitamin B_{12}, choline and brewers' yeast. While they found that liver extract was the most effective in returning liver fat to normal they state that the yeast supplement (0.25 gm per rat per day) offered complete protection when the rats were fed a 6% protein diet and was partially effective when the diet contained 16% protein. The rats in the present study were fed a diet containing 18% casein and 4% brewers' yeast. On the basis of 10 gm of food consumption per rat per day they received about 0.40 gm of brewers' yeast per day. Though no liver fat analyses were made on the rat livers analyzed for vitamin A in this study and Drill ('54) showed no vitamin A analyses on the livers on which they reported liver fat, it is of interest that the use of strictly synthetic compounds, vitamin B_{12}, choline and folic acid did not in either case bring about the same results as when the brewers' yeast was fed. In the present study greater storage of vitamin A was obtained when vitamin B_{12} was fed in conjunction with the yeast-containing diet than when it was fed the synthetic-vitamin-mix diet.

Cholesterol

Serum cholesterol. Female rats. The serum cholesterol of rats receiving no choline for 18 days, group 1, was not significantly different from that of rats receiving the various levels of choline, groups 2 through 6, table 1. However, when the first three groups of rats receiving 0, 30 and 60 mg of choline per 100 gm of diet were compared with groups 4, 5 and 6 receiving 100, 250 and 350 mg of choline, the rats receiving the lower levels of choline had significantly less serum cholesterol than did those receiving the higher levels ($P < 0.01$).

Feeding 3000 mμg of vitamin B_{12} to female rats in group 7 receiving the synthetic-vitamin-mix diet caused a significant lowering of serum cholesterol ($P < 0.05$) when compared with group 5 receiving the same amount of choline but no vitamin B_{12}. The serum cholesterol of rats in group 8 receiving the unsupplemented yeast diet was similar to that in group 2 fed the synthetic-vitamin-mix diet with the same amount of choline, and group 9, receiving 350 mg of choline in the yeast diet, was similar to group 6 fed the synthetic-vitamin-mix diet with 350 mg of choline.

The addition of either 350 mg of choline or 3000 mμg of vitamin B_{12} to the yeast diet brought about significantly higher serum cholesterol values than when the unsupplemented yeast diet was fed, group 9, 10 and 8 respectively.

Male rats. The response of the male rats to the diets and supplements tested was dissimilar to that of the female rats in that: rats in group 1, receiving no choline, had lower ($P < 0.05$) serum cholesterols than did those in groups 2 through 6 receiving the various levels of choline; the addition of vitamin B_{12} to the synthetic-vitamin-mix diet did not have a significant effect on serum cholesterol, group 7 versus group 5; and male rats receiving the unsupplemented yeast diet (group 8) had lower serum cholesterol ($P < 0.01$) than did those in group 2 receiving the synthetic-vitamin-mix diet with the same amount of choline.

As with the female rats, the males in the first three groups on the smaller levels of choline had lower serum cholesterol

than did those in groups 4, 5 and 6, fed the larger amounts of choline; the addition of either 350 mg of choline or 3000 mμg of vitamin B_{12} to the yeast diet brought about significantly higher serum cholesterol values than when the unsupplemented yeast diet was fed, groups 9, 10 and 8 respectively; and rats in group 9 receiving 350 mg of choline in the yeast diet had serum cholesterols similar to those in group 6, fed the synthetic-vitamin-mix diet with 350 mg of choline.

Liver cholesterol. Female and male rats. Both male and females rats receiving no choline in their diet for 18 days, group 1, had more total cholesterol ($P < 0.05$) expressed as percentage of the moist weight of the liver than did those in groups 2 through 6 receiving the various levels of choline, table 1. Feeding 3000 mμg of vitamin B_{12} to male or female rats in group 7 receiving the synthetic-vitamin-mix diet, or in group 10 receiving the yeast diet did not affect the liver cholesterol. It would appear that the male rats receiving the unsupplemented yeast diet, group 8, had less cholesterol in their livers than did the male rats in group 2 fed the synthetic-vitamin-mix diet with the same amount of choline. However, this difference was not statistically significant with an F value of 3.83 and an F value of 4.04 necessary for significance at 0.05. Any other differences between the yeast-containing diets and the synthetic-vitamin-mix diets were not statistically significant.

Correlation: Serum and liver cholesterol versus vitamin A storage. Further analysis of the data showed no correlations between storage of vitamin A in the liver and serum or liver cholesterol in either the male or female rats or between serum and liver cholesterol. Bring, Warnick and Woods ('55) reported a highly significant positive correlation between serum vitamin A and serum total cholesterol in 15- and 16-year-old school children. No reports could be found in the literature giving values for vitamin A in rat liver together with co-occurring serum and liver cholesterol. When the rats used in this study were fed the experimental diets together with the carotene, choline and vitamin B_{12} supplements for

14 days, there was no indication of a relationship between the rat's ability to utilize carotene and store vitamin A and the co-occurring serum and liver cholesterol values.

Discussion: Serum and liver cholesterol. The mean total cholesterols in the serum of all groups of rats, with the exception of the males in group 8 receiving the unsupplemented yeast diet, are somewhat higher than 70 mg % reported by Ridout, Patterson, Lucas and Best ('54) as the mean for young adult rats on a stock ration and also higher than those reported by Page and Brown ('52) for three groups of normal rats, 67, 75 and 82 mg %. Serum cholesterol values from stock rats in this laboratory have ranged from 70 to 90 mg % of cholesterol.

Although the effect of dietary choline on mean serum cholesterol values is not marked and in some cases not statistically significant, the results in general are in agreement with the findings of other workers as expressed by Ridout et al. ('54). They state that supplementary choline increases the serum cholesterol in omnivorous animals fed hypolipotropic diets containing cholesterol and that consideration of all the available data indicates that there is no general agreement that choline has any specific influence on serum cholesterol in man or in experimental animals receiving adequate diets.

The mean total cholesterol in the liver of rats in group 1 receiving no dietary choline for approximately 18 days, 0.45% moist weight of liver, is similar to the value of 0.42 calculated from the data shown by Ridout, Lucas, Patterson and Best ('52) for rats receiving no dietary choline for 21 days. However, the mean liver cholesterols reported by Ridout et al. for rats receiving 80 or 320 mg of choline per 100 gm of diet, 0.28 and 0.27% of the moist weight of liver, are somewhat lower than the range of mean values, 0.31 to 0.40, obtained in the present study when the levels of choline fed were 30, 60, 100, 250 and 350 mg per 100 gm of diet or when the diet contained added vitamin B_{12}. The males in group 8, receiving the unsupplemented yeast diet and those in group 9, fed the same diet with 350 mg of choline, with liver cholesterols of 0.31% of the

moist weight approach the values reported by Ridout et al. All liver cholesterol values obtained in the present study are slightly higher than the means of three groups of normal rats reported by Page and Brown ('52), 0.177, 0.265 and 0.205, and a mean obtained in this laboratory of 0.27% of the moist weight for 12 male and female rats fed a normal stock diet [13] for 120 days.

SUMMARY

The feeding of 0, 30, 60, 100, 250 or 350 mg of choline or 3000 mμg of vitamin B_{12} per 100 gm of diet to male or female rats receiving a synthetic-vitamin-mix diet and 60 μg of carotene daily for 14 days did not change the rats' utilization of carotene and the resulting liver and kidney storage of vitamin A.

Male rats fed the yeast diet with 350 mg of choline per 100 gm of diet stored significantly more vitamin A than did those receiving the synthetic-vitamin-mix diet with 350 mg of choline ($P < 0.01$), or more than those fed the unsupplemented yeast diet with 30 mg of choline from the yeast ($P < 0.01$). These differences were not significant for the females.

Both male and female rats receiving the yeast-containing diet supplemented with 3000 mμg of vitamin B_{12} per 100 gm of diet stored significantly more vitamin A than did those receiving the unsupplemented yeast diet ($P < 0.01$) or those fed the synthetic-vitamin-mix diet with 3000 mμg of vitamin B_{12} ($P < 0.01$).

The results indicate the presence in yeast of some factors (or factor) which, together with vitamin B_{12}, but not alone, bring about greater utilization of carotene and larger storage of vitamin A in the livers and kidneys of rats than when vitamin B_{12} is fed with a synthetic-vitamin-mix diet. It is also suggested that the protein composition of the yeast may be such that the amino acid balance of the yeast containing diet when supplemented with vitamin B_{12} but not alone, provides for greater utilization of carotene.

[13] Purina Laboratory Chow, Purina, Mills, St. Louis 2, Missouri.

The serum cholesterol of female rats receiving no choline for 18 days was not significantly different from that of rats receiving 30, 60, 100, 250 or 350 mg of choline per 100 gm of diet. Male rats receiving no choline had significantly lower serum cholesterols than did those receiving the choline supplements ($P < 0.05$). Both male and female rats receiving 0, 30 or 60 mg of choline per 100 gm of diet had lower serum cholesterols than did those receiving the higher levels of choline 100, 250 or 350 mg ($P < 0.01$).

Feeding 3000 mμg of vitamin B_{12} to female rats receiving 250 mg of choline and a synthetic-vitamin-mix diet caused a significant lowering of serum cholesterol when compared with that of females receiving the same amount of choline but no vitamin B_{12}. Male rats did not respond in a like manner to the feeding of vitamin B_{12}.

Male rats receiving the unsupplemented yeast diet had lower serum cholesterol than did those receiving the synthetic-vitamin-mix diet with the same amount of choline ($P < 0.01$). This difference was not significant for the females.

Male and female rats receiving no choline in their diet for 18 days had more total cholesterol in their livers than did those rats receiving either 30, 60, 100, 250 or 350 mg of choline per 100 gm of diet.

The addition of 3000 mμg of vitamin B_{12} per 100 gm to the synthetic-vitamin-mix diet or the yeast-containing diet did not affect the liver cholesterol of male or female rats.

There were no correlations between storage of vitamin A in the liver and serum or liver cholesterol or between serum and liver cholesterol.

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COMPARISON OF METABOLIZABLE ENERGY AND PRODUCTIVE ENERGY DETERMINATIONS WITH GROWING CHICKS

F. W. HILL AND D. L. ANDERSON [1]
Agricultural Experiment Station and Graduate School of Nutrition
Cornell University, Ithaca, New York [2]

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Accurate data on the energy values of purified nutrients and crude feeding materials are a basic need for quantitative studies in energy nutrition. Presently available information pertaining to avian species is of three kinds. Extensive digestibility data have been summarized by Olsson ('50) and Fraps ('44) for most common feeding materials, and are expressed as digestibility coefficients for protein, fat, crude fiber and nitrogen-free extract. A limited number of directly determined metabolizable energy values are available, and are contained in the reports of Fraps et al. ('40), Olsson ('50), Halnan ('51) and Carpenter and Clegg ('56). Productive energy, an estimate of net energy based on a carcass analysis technique using growing chicks, is a measure that has been employed by Fraps ('46) for the estimation of net energy values of many feedstuffs.

For most purposes, metabolizable energy and net energy are the measures of greatest utility. Digestibility data are subject to the limitations of methods used for chemical or physical separation of feces and urine in mixed excreta, or the difficulties of using surgically altered animals for the separate collection of fecal and urinary wastes. Because of these limitations, the computation of metabolizable energy values from

[1] Present address, Department of Poultry Husbandry, University of Massachusetts, Amherst, Massachusetts.

[2] These studies were conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

available digestibility data is not completely satisfactory, although useful information has been obtained in this way by Axelsson and Ericksson ('51) and Titus ('55). Furthermore, some of the samples used in early experiments are not representative of present day feeding materials. Although in theory some measure of net energy would be the most valuable criterion of energy value, the uncertainty of net energy measurements with other species makes it desirable to obtain confirmatory evidence to supplement the data of Fraps for chickens. The purpose of the experiments to be described was to compare metabolizable enery and productive energy determinations using growing chicks in order to establish which of these measures is the most useful for quantitative studies in energy nutrition of the chick and for evaluation of feeding materials.

MATERIALS AND METHODS

All of the experiments to be described were conducted using diet E9, a semi-purified reference diet the composition of which is shown in table 1. The diet was formulated to be adequate in all recognized nutrient requirements of the chick, and liberally fortified with respect to unidentified nutrients as supplied by fish meal, fish solubles, brewers' dried yeast and dried whey. Male cross-bred (RIR $\times$ BPR) chicks were used in all experiments. They were reared to 9 or 10 days of age on diet E9 and were allotted to groups of 10 chicks at this time by a procedure similar to that of McKittrick ('47) to equalize both body weight and rate of gain among experimental groups. The length of the experimental period was 14 days following the formation of the groups.

Productive energy was determined by a method similar to that of Fraps ('46) excepting that each treatment was applied to a group of 10 chicks rather than to individual chicks. The Fraps procedure is based on the difference in energy gain produced by feeding a given diet at two planes of food intake, normally 100% and 50% of ad libitum, using the following expression to compute productive energy: $WM + G = FX$.

in which W is the average chick weight by periods computed from the weights at the beginning (W_1) and after 7 and 14 days of experiment (W_2, W_3) by the expression $\frac{W_1 + 2W_2 + W_3}{4}$; M is the maintenance requirement per unit weight; G is the energy gain computed from carcass compostion of the experimental lots and a lot sacrificed at the beginning of the experimental period; F is the food intake; and X equals productive energy value of the diet per unit weight. Observations on each

TABLE 1

Composition of diet E9

COMPONENT	PER 100 GM	COMPONENT	PER 100 GM
	gm		*gm*
Glucose [1]	44.1	Fish solubles (dry basis)	1.0
Ground wheat	9.0	Hydrogenated vegetable fat [2]	2.5
Soybean oil meal		Ground limestone	2.0
(44% protein)	17.5	Dicalcium phosphate	1.0
Crude casein	10.5	Salt (iodized)	0.5
Gelatin	2.5	Mineral mixture [3]	0.4
Fish meal (menhaden)	4.0	Vitamin mixture [3]	0.5
Dried brewers' yeast	2.5		
Dried whey	2.0		100.0

[1] Cerelose.

[2] Hydora, Lever Bros.

[3] The mineral and vitamin mixtures supply, in milligrams per 100 gm of diet: 220 K_2HPO_4, 120 $MgSO_4$, 30 $MnSO_4$, 30 $FeSO_4 \cdot 7H_2O$, 0.8 $CuSO_4 \cdot 5H_2O$, 0.3 thiamine, 0.4 riboflavin, 1.0 calcium pantothenate, 0.5 pyridoxine, 2.6 niacin, 0.07 folacin, 0.09 menadione, 0.01 biotin, 0.001 vitamin B_{12}, 130 choline chloride, 1000 USP units vitamin A, 100 I.C. units vitamin D_3, 2.2 mg α-tocopheryl acetate.

lot supplied data for W, G and F, leaving M and X as unknowns. Simultaneous equations for the two lots of chicks fed at different planes of intake permit solving the expressions for X, on the assumption that maintenance requirement per unit weight is essentially constant over the weight range concerned, and that productive energy value of the diet is the same at the two planes of intake.

As conducted in this laboratory, the determinations included analysis of initial body composition using a representative group of 10 chicks sacrificed at the beginning of the experi-

mental period. The small differences in average initial weight among the experimental groups were compensated for in estimating their initial composition by assuming that absolute composition and average weight were proportional over this narrow range. Final composition was determined by sacrificing each experimental group at the end of the 14-day experimental period. The chicks were killed by dislocating the neck without blood loss, the contents of the digestive tract were removed and the carcasses frozen. After passing them through a meat grinder, the carcasses for each lot were dried from the frozen state in a large freeze-drying apparatus. The dried material was equilibrated with atmospheric moisture and ground in a Wiley mill, after which it was thoroughly mixed, sampled and analyzed for moisture, fat (ether extract) and protein ($N \times 6.25$). Successive weighings at each step of the preparation procedure were made so that mechanical losses were accounted for and the absolute amounts of fat, protein and energy were determined for each lot. Energy gains were computed from fat gain at 9.35 Cal. per gram, and protein gain at 5.66 Cal. per gram, as described by Fraps, and also by other procedures as discussed later.

Metabolizable energy values were determined by using a composite of mixed excreta from each lot collected on the last three consecutive days of the experimental period. The excreta were frozen after each collection and pooled before drying. Chromic oxide at a level of 0.2% of the diet was used as an index substance in order to eliminate the need for quantitative collections of excreta and quantitative measurement of feed intake (Schürch, Lloyd and Crampton, '50; Dansky and Hill, '52). Analyses were made on feed and excreta as follows:

Moisture: by air oven or vacuum oven.

Nitrogen: by macro-Kjeldahl procedure.

Combustible energy: determined in a Parr adiabatic oxygen bomb calorimeter.

Chromic oxide: determined spectrophotometrically following wet ashing according to the following procedure: a sample

containing approximately 10 mg of chromic oxide is transferred to a 100 ml Kjeldahl flask calibrated at 110 ml. Addition of 10 ml of concentrated nitric acid and allowing the mixture to stand overnight makes subsequent digestion easier, but this step is not necessary; analyses in the experiments reported in this paper were made without benefit of pre-digestion, but more recent work in this laboratory has generally employed it. Where nitric acid is used, the mixture is heated over a micro-burner to dryness, and 15 ml of digestion mixture added. The digestion mixture is prepared by dissolving 10 gm of sodium molybdate in 150 ml of distilled water and slowly adding 150 ml of concentrated sulfuric acid; after cooling in an ice bath, 200 ml of 70% perchloric acid are added with stirring. Digestion of the sample is continued over the open flame of a micro-burner until the green color changes to yellow, orange or red, depending on chromium concentration. The flask is immediately chilled in cold water and diluted with approximately 90 ml of distilled water. After cooling, the mixture is brought to volume (110 ml) and allowed to stand overnight to precipitate inorganic matter. Optical density is determined at 430 mμ in a Beckman DU spectrophotometer. Chromic oxide content is computed from a reference curve covering the range 0 to 150 μg of chromic oxide equivalent per milliliter prepared by aliquot dilutions from digestion of 50 mg of pure chromic oxide as outlined above. In our laboratory the linear reference curve has the form $X = 0.3454\ Y$ where X = milligrams chromic oxide equivalent per milliliter, and Y = optical density.

Metabolizable energy was computed as follows from the analytical data, all expressed on a dry matter basis:

E_{diet} = Calories combustible energy per gram of diet dry matter (determined directly by bomb calorimeter)

$E_{excreta}$ = Calories combustible energy in excreta per gram of diet dry matter

$$= \text{Calories per gram excreta} \times \frac{Cr_2O_3 \text{ per gram diet}}{Cr_2O_3 \text{ per gram excreta}}$$

N = Nitrogen retention per gram of diet dry matter

$$= \text{N per gram diet} - \text{N per gram excreta} \times \frac{Cr_2O_3 \text{ per gram diet}}{Cr_2O_3 \text{ per gram excreta}}$$

$$\text{Metabolizable energy per gram diet dry matter} = E_{diet} - E_{excreta} - 8.22\ N$$

In the computations summarized above, correction for positive nitrogen balance was made in order to convert all data to a basis of nitrogen equilibrium for comparative purposes. It was assumed that protein tissue if oxidized for energy purposes would yield uric acid as the sole excretory product, and the value 8.22 used above is the combustible energy value of uric acid per gram of nitrogen. This assumption is not strictly correct because normal chicken urine contains only 60 to 80% of nitrogen as uric acid (Katayama, '24; Coulson and Hughes, '30). However, the error in this assumption is probably not great since calculation of the energy value of chicken urine per gram of nitrogen from the data of Coulson and Hughes ('30) yields a value of 8.7 Cal. per gram of urinary nitrogen; furthermore, the assumption that oxidation of body tissue would yield the same pattern of nitrogen excretion products as normally found in the urine of chickens appears to be no better than the assumption that all the nitrogen is excreted as uric acid. In any case, the range of choice is small and the simplest assumption appears preferable.

EXPERIMENTAL

The first two experiments were conducted to determine the effect of plane of food intake on metabolizable energy and productive energy value of the reference diet. This was considered desirable because no such studies in relation to metabolizable energy determinations with chickens are known to the authors, and the effect of plane of nutrition on productive energy estimations had not been investigated by Fraps. It appeared possible that there may be a limiting level of food intake below which accurate estimation of energy value cannot be made because of physiological disturbances. A single lot of 10 chicks was used for each treatment in each of these experiments. In the first experiment, levels of food intake ranging from 100% to 50% of ad libitum by 10% steps were used; in the second experiment, the 90% ad libitum group

was omitted and two additional groups were fed 40% and 30% of ad libitum consumption respectively.

The results of these experiments are presented in tables 2 and 3. Productive energy was calculated by simultaneous equations as described above, pairing the data for each restricted lot with the ad libitum lot of the respective experiment. The productive energy values showed a wide range of variation both between levels of food restriction and within the same treatment in replicate experiments. It was evident that restriction of food intake to 90% of ad libitum did not produce a sufficiently large difference in energy gain and food intake to permit accurate estimation of productive energy. At the other levels of restriction, there was no evident relationship between plane of intake and productive energy value. On the basis of these results, it was concluded that any plane of restriction less than 80% of ad libitum would be sufficient to produce accurately measurable differences in energy gain and feed intake as compared to ad libitum feeding.

In contrast to the highly variable results obtained in the estimation of productive energy, the metabolizable energy value of diet E9 was notably constant within each replicate experiment. There was no apparent relationship between plane of intake and the metabolizable energy value per unit weight of diet. The difference between the average values in the two replicate experiments, approximating 4% of the mean value, was probably due to small differences in the composition of the diet in the two experiments because the diet was formulated on an air-dry rather than dry-matter basis.

On the basis of these findings, 6 additional experiments were conducted to determine the precision of measurement of the productive energy and metabolizable energy values for diet E9. The two planes of food intake chosen were 100% and 60% of ad libitum. The data from these experiments and the corresponding data from the first two are summarized in

TABLE 2

Effect of plane of feed intake on determination of metabolizable energy and productive energy

(Experiment 1)[1]

PERCENTAGE OF AD LIBITUM FEED INTAKE	AVERAGE WEIGHT		AVERAGE GAINS			AVERAGE FEED CONSUMED [2]	ENERGY VALUE OF DIET [2]	
	Initial	Final	Protein	Fat	Energy		Productive	Metabolizable
	gm	*gm*	*gm*	*gm*	*Cal.*	*gm*	*Cal./gm*	*Cal./gm*
100	95.8	300.1	37.0	20.6	402.0	313	—	3.30
90	94.9	282.5	30.9	14.1	306.7	282	4.76	3.25
80	96.7	263.7	28.2	12.0	271.5	251	2.79	3.23
70	94.9	247.7	22.2	7.2	193.0	222	3.24	3.23
60	95.9	216.1	20.8	3.5	150.8	189	2.85	3.26
50	93.1	186.9	16.8	1.0	104.6	158	2.84	3.25
						Average:	3.30	3.25

[1] Experimental period from 9 to 23 days of age. Lot sacrificed at 9 days to provide initial data had following composition: average weight 97.5 gm, protein 15.1 gm, fat 7.3 gm.

[2] Expressed on dry matter basis.

TABLE 3

Effect of plane of feed intake on determination of metabolizable energy and productive energy

(Experiment 2)[1]

PERCENTAGE OF AD LIBITUM FEED INTAKE	AVERAGE WEIGHT		AVERAGE GAINS			AVERAGE FEED CONSUMED [2]	ENERGY VALUE OF DIET [2]	
	Initial	Final	Protein	Fat	Energy		Productive	Metabolizable
	gm	*gm*	*gm*	*gm*	*Cal.*	*gm*	*Cal./gm*	*Cal./gm*
100	105.8	337.4	41.5	20.9	430.2	343	—	3.38
80	106.4	284.4	32.5	15.2	326.4	270	1.61	3.42
70	102.5	248.4	27.1	8.3	231.0	237	3.02	3.38
60	103.7	234.2	25.5	6.5	205.2	203	2.06	3.39
50	105.4	204.8	20.4	1.9	133.2	170	2.36	3.39
40	102.3	176.1	15.4	—1.8	70.6	136	2.52	3.37
30	105.2	142.1	10.4	—4.2	19.8	102	2.46	3.39
						Average:	2.34	3.39

[1] Experimental period from 9 to 23 days of age. Lot sacrificed at 9 days to provide initial data had following composition: average weight 104.1 gm, protein 16.5 gm, fat 8.5 gm.

[2] Expressed on dry matter basis.

TABLE 4

Summary of eight replicate determinations of metabolizable energy and productive energy of diet E 9

EXPERIMENT	AVERAGE WEIGHT		AVERAGE GAINS			AVERAGE FEED CONSUMED [1]	ENERGY VALUE OF DIET	
	Initial	Final	Protein	Fat	Energy		Metabolizable	Productive
	gm	*gm*	*gm*	*gm*	*Cal.*	*gm*	*Cal./gm*	*Cal./gm*
1	95.8	300.1	37.0	20.6	402.0	313	3.30	2.85
	95.9	216.1	20.8	3.5	150.8	189	3.26	
2	105.8	337.4	41.5	20.9	430.2	343	3.38	2.06
	103.7	234.2	25.5	6.5	205.2	203	3.39	
3	123.2	346.4	41.7	22.2	443.5	338	3.33	2.33
	125.2	247.1	24.6	7.1	205.4	203	3.34	
4	104.3	322.7	40.2	21.6	429.9	331	3.38	2.44
	109.5	227.7	24.0	5.1	183.1	200	3.37	
5	116.5	329.5	41.1	20.1	421.1	337	3.29	2.26
	117.5	248.3	24.6	6.4	199.2	209	3.40	
6	115.3	359.9	44.0	25.7	489.2	378	3.29	3.31
	113.9	226.8	23.8	3.2	164.5	220	3.36	
7	97.1	290.7	35.0	18.2	368.3	305	3.31	2.58
	100.3	193.7	19.0	3.6	141.1	179	3.38	
8	96.4	310.9	38.1	20.8	410.2	331	3.32	2.13
	98.2	214.3	22.3	5.8	180.2	193	3.40	
						Average $\pm \sigma$	3.34 $\pm$ 0.045	2.49 $\pm$ 0.42

[1] The two lots in each experiment were fed ad libitum and 60% of ad libitum respectively.

table 4 and show that the productive energy ranged from 2.06 to 3.31 Cal. per gram of dry matter with a mean of 2.49 $\pm$ 0.42 (standard deviation). In marked contrast to the variability of replicate estimations of productive energy, the metabolizable energy of diet E9 was highly consistent in replicate experiments. The 8 estimates based on ad libitum

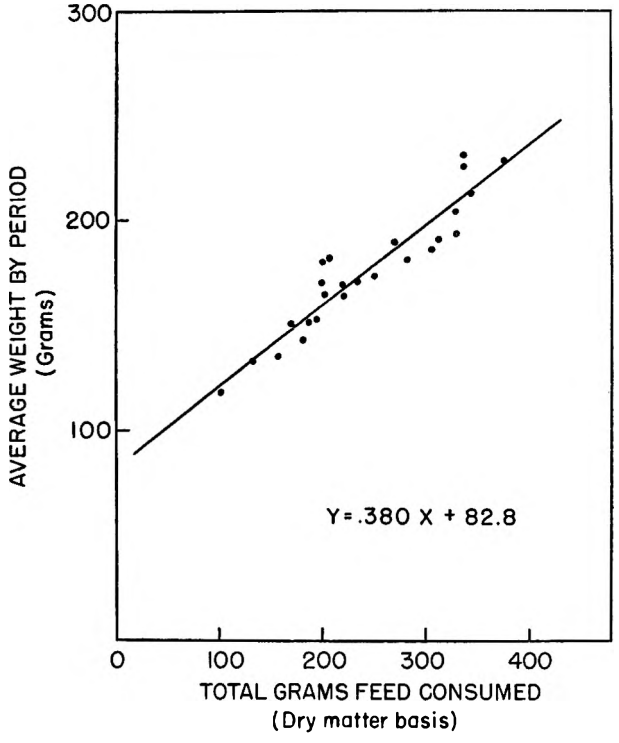

Fig. 1 Regression of average weight by period (Y) on total feed consumed (X) for determining productive energy of diet E9.

feeding averaged 3.32 Cal. per gram, while at restricted intake they averaged 3.36 Cal. per gram. The difference between the values obtained at the two planes of intake was not significant. The over-all mean was 3.34 $\pm$ 0.045 Cal. per gram.

To obtain a more valid estimate of the productive energy value of diet E9 than a simple mean of replicate determina-

tions, use was made of a regression analysis technique. The data from all of the experiments presented in tables 2, 3 and 4 were used to compute the regressions of energy gain on total feed consumption and of average weight during the experiment on total feed consumption. The data and the computed

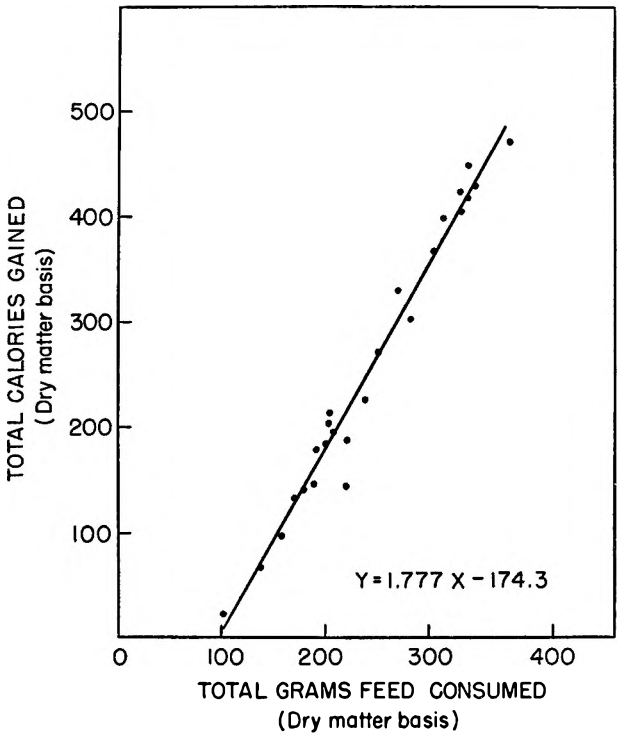

Fig. 2 Regression of total Calories gained (Y) on total feed consumed (X) for determining productive energy of diet E9.

regression lines are shown graphically in figures 1 and 2 together with the linear equations derived for the indicated relationships. By selecting two arbitrary rates of feed consumption, mean values for energy gain and average weight were derived from these regressions for each plane of intake and inserted into the equations previously described for computation of productive energy. This procedure yielded value

of 2.58 Cal. of productive energy per gram of dry matter in diet E9.

DISCUSSION

From the data of these experiments it is clearly evident that metabolizable energy is a highly precise measure of food energy for the chick, and that the estimation of productive energy is associated with a high degree of variation. It is perhaps not surprising that this should be a characteristic of productive energy, since estimations of net energy with other species have also been highly variable.

It is significant to note that the productive energy value obtained for the reference diet in this work was approximately 75% of its metabolizable energy. The large difference between the two measures of energy value, attributable presumably to specific dynamic action (SDA), is considerably greater than would be expected from work with other animals (Swift and French, '54). Whether it represents a distinctively high SDA characteristic of the growing chick or a bias in the method for determining productive energy cannot be deduced from the present data. It should be considered, however, whether two basic assumptions on which the Fraps method is based may be responsible, at least in part. It is assumed that the productive energy value per unit weight of diet is unaffected by plane of food intake. From work with other species, it is considered probable that net energy value is affected by plane of nutrition; the absence of any consistent relation between rate of restricted food intake and the productive energy of the diet in the present work does not necessarily conflict with this view, since the effect of restriction may already have been maximal at a reduction of 20 to 30% below ad libitum consumption.

A further assumption in the Fraps procedure is that maintenance requirement per unit body weight is constant over the range of body weights concerned. It is of course well established that basal metabolic rate and total maintenance

requirement are exponential functions of body weight rather than linear ones. The effect of using a body weight term to the 0.7 power rather than average weight by periods in the simultaneous equations for computing productive energy is shown in table 5, which gives the arithmetic mean and standard deviation for calculations based on the data from the 8

TABLE 5

The effect of using an exponential function of body weight and different energy equivalents for tissue protein in the computation of productive energy of diet E 9

EXPRESSION OF BODY WEIGHT	PRODUCTIVE ENERGY PER GRAM OF DIET E 9	
	Assigning tissue protein 5.66 Cal. per gm	Assigning tissue protein 4.34 Cal. per gm
	Cal./gm	*Cal./gm*
Average weight by periods $\left(\frac{W_1 + 2W_2 + W_3}{4}\right)$	2.49 ± 0.42 (17) [1]	2.32 ± 0.40 (17)
Exponential average weight by periods $\left(\frac{W_1 + 2W_2 + W_3}{4}\right)^{0.7}$	2.18 ± 0.28 (13)	2.01 ± 0.27 (14)
Average of initial and final weights $\left(\frac{W_1 + W_3}{2}\right)$	2.56 ± 0.52 (20)	2.38 ± 0.50 (21)
Exponential average of initial and final weights $\left(\frac{W_1 + W_3}{2}\right)^{0.7}$	2.21 ± 0.31 (14)	2.04 ± 0.31 (15)

[1] Mean ± standard deviation (coefficient of variation, $\frac{100\,\sigma}{M}$).

replicate determinations described in table 4. This change in computation has the effect of reducing the productive energy estimate, and therefore increasing the difference between it and metabolizable energy. If the large SDA effect is an artifact, it is evidently not due to the use of a linear rather than exponential function of body size in estimating maintenance cost. Also shown in table 5 is the effect of using the mean of initial and final weights rather than average

weight by periods; this tended to increase slightly the numerical value of the productive energy estimate.

The use of the factor 5.66 Cal. per gram to compute the energy equivalent of protein gain may also be questioned in this connection. Since the net energy value of tissue protein when oxidized should be no more (and probably less) than its metabolizable energy, it may be incorrect to credit protein with its full heat of combustion in computing energy gain of growth. Making the simplifying assumption that the end product of tissue protein oxidation would be uric acid, the heat of combustion of which is 8.22 Cal. per gram of nitrogen, the metabolizable energy yield per gram of tissue protein may be estimated at $5.66 - 0.16 \times 8.22 = 4.34$ Cal. per gram. The effect of using this factor to compute energy gain instead of 5.66 is also shown in table 5, for both the linear and exponential expressions of body weight. This results in a reduction of the numerical value of the productive energy estimate in every case, so the large difference between productive and metabolizable energy is evidently not due to use of the factor 5.66 for protein energy.

Although these alternative methods of computing productive energy give no indication of a reason for the large apparent SDA factor in energy utilization by the chick, it is of interest to note their effect on the variation as well as the magnitude of productive energy estimates. Using the average of initial and final weights instead of average weight by periods increased productive energy value slightly. It also increased variability as measured by the coefficient of variation, substantially when a linear expression of weight was used and slightly when an exponential expression was used. Using the factor 4.34 instead of 5.66 Cal. per gram of protein increased variability slightly as it reduced the numerical value of productive energy. Employing the exponential expression for relating maintenance need to body weight reduced the variation substantially (3 to 6%) and also reduced size of the productive energy estimate.

If the large difference between productive energy and metabolizable energy is indeed a valid estimate of SDA, this factor is a large part of the total heat production of the chick. If, as would seem more likely, productive energy is a parameter of net energy rather than a true estimate of it, the numerical value of the difference between productive and metabolizable energy would have little meaning in itself. Its biological significance, as well as that of the productive energy estimate itself, is uncertain.

It is also of interest to note that the mean productive energy value obtained in this work, 2.58 Cal. per gram, is approximately 20% greater than estimated for this diet from the data of Fraps for the diet components. The greater productive energy observed in these studies may reasonably be considered to be due to better nutritive balance and greater adequacy in micronutrients of our diet in comparison to those used in the earlier studies of Fraps.

These experiments indicate that metabolizable energy is the measure of choice for chicks with respect to accuracy. Whether it is a valid measure of metabolically useful energy, equally as valuable as some measure of net energy, will be the subject of further experiments.

SUMMARY

Experiments have been conducted to compare the determination of metabolizable and productive energy of a semi-purified reference diet for the growing chick.

Metabolizable energy was found to be independent of plane of food intake in the range from 100 to 30% of ad libitum intake. Replicate determinations gave a mean value of 3.34 Cal. per gram of dry matter with standard deviation $\pm$ 0.04.

In contrast, replicate determinations of productive energy were highly variable, ranging from 2.06 to 3.31 Cal. per gram with a mean of 2.49 $\pm$ 0.42. From a regression analysis of all available data, its mean productive energy was 2.58 Cal. per gram. No consistent relationship between plane of food intake and productive energy value was found.

The observed productive energy was approximately 20% greater than estimated from the productive energy data of Fraps for the components of the diet. This was considered to be due to the superiority of the diet used in the present work as compared to the earlier studies of Fraps.

Productive energy in the present experiments was approximately 77% of metabolizable energy. The large difference between the two measures was not diminished by changes in method of computation involving the relation between body size and maintenance requirement or the energy equivalent of tissue protein. Its possible significance was discussed.

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ADDENDUM

The work of J. Davidson, I. McDonald and R. B. Williams described in a paper entitled "The utilization of dietary energy by poultry. I.-A study of the algebraic method for determining the productive energy of poultry feeds" (J. Sci. Food Agric., *8:* 173–182, '57) came to our attention after the preparation of our manuscript. In their studies the productive energy determination was also highly variable, and they obtained values for simplified rations which were 10 to 40% lower than those predicted by Fraps' data. They concluded that the assumptions underlying the method are invalid, and that the values obtained are unreliable.

EFFECTS OF EXERCISE ON BLOOD (PLASMA) CONCENTRATIONS OF VITAMIN A, CAROTENE AND TOCOPHEROLS

ROBERT W. HILLMAN AND MARTIN C. ROSNER
WITH THE TECHNICAL ASSISTANCE OF MARIE E. LEOGRANDE
Department of Environmental Medicine and Community Health, State University of New York College of Medicine at New York City, Brooklyn

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Vitamin concentrations in the blood plasma are affected by many factors other than the nutritional state (Popper and Steigmann, '43; Popper et al., '49; Darby et al., '49; Moore and Sharman, '51; Urbach et al., '52; Hillman et al., '55a). Physical stress sometimes seems important among these influences (Urbach et al., '52).

James and ElGindi ('53) and James et al. ('53) described increased plasma vitamin A and carotene levels induced by physical exercise. Widely varying changes observed in track athletes appeared related to the intensity and duration of exertion, and to the physical condition of the subjects. Short periods of exertion tended to depress rather than elevate the plasma concentrations of these substances, especially in subjects in advanced stages of training. Fasting, pre-exercise levels were generally higher at the end than at the beginning of the training period.

The present study was designed to measure the blood plasma vitamin A and carotene responses to a standardized physical stress — the "Harvard" two-step test, which has been widely adapted to the evaluation of physical fitness. The plasma tocopherol response was also determined on the basis of its metabolic relationship to vitamin A (James et al., '53; Hickman et al., '42; Moore, '45; Van Bruggen and Straumfjord, '48) and the low blood concentration reported following severe exertion (Urbach et al., '52).

MATERIAL AND METHODS

The subjects were 14 males, ages 22 to 40, medical students and staff personnel in apparent good health, but in varying degrees of physical fitness. A total of 30 tests was performed, including 14 on one and two on each of three other subjects. Six tests were in the post-absorptive state; 24 were performed two to 5 hours after a meal. The 20-inch step-up test was employed, as devised by Johnson et al. ('42), modified to a rate of 20 to 30 step-ups per minute (average 25), for as long as the subject could continue, up to a maximum of 5 minutes.[1] The pulse rate was counted over a period of 30 seconds, at 15 minutes and immediately before exercise, and at one, two and three minutes after its completion. The last three values were combined to calculate the "Brouha" Index (the total of these readings divided into the duration of exercise in seconds, times 50).

Blood samples for vitamin A, carotene and tocopherol determinations were drawn 15 minutes and again immediately before exercise, and at intervals of 5, 20 and 60 minutes following the test period. Vitamin A was determined by the method of Bessey et al. ('46) adapted to 3-ml samples. Tocopherol was measured by a macro-adaptation of the method of Quaiffe et al. ('49), entailing the preliminary estimation of carotene. In most instances determinations were done in duplicate. Insufficient plasma precluded the determination of vitamin A levels in one, and carotene and tocopherol concentrations in another case, respectively.

RESULTS

The overall plasma concentrations of vitamin A, carotene and tocopherol are indicated in table 1 and the accompanying figure.

[1] Individual work load is not fully equated by use of a constant height step. Factors of body weight and limb length represent uncompensated variables, which may be as important as the rate of step-up, especially among untrained, largely sedentary subjects.

TABLE 1

Mean plasma levels of vitamin A, carotene and tocopherol, before and after the two-step test (14 subjects, 30 tests)

SUBSTANCE	SPECIMEN PARAMETER [1]	1	2	3	4	5	1, 2	3, 4, 5
Vitamin A, μg%	Mean	68	71	72	79	75	70	75
	S.D.	21.0	24.0	26.6	27.3	24.8	22.9	23.5
	C.V.	31	34	37	35	33	33	31
	S.E.	3.9	4.4	4.9	5.1	4.6	4.2	4.4
Carotene, μg%	Mean	102	99	106	98	89	100	98
	S.D.	33.6	29.1	36.9	37.9	32.7	32.4	18.5
	C.V.	33	29	35	39	37	32	19
	S.E.	6.2	5.4	6.8	7.0	6.1	6.0	3.4
Tocopheral, mg%	Mean	0.83	0.72	0.80	0.70	0.62	0.77	0.71
	S.D.	0.39	0.39	0.39	0.42	0.38	0.41	0.39
	C.V.	47	54	49	60	61	53	55
	S.E.	0.07	0.07	0.07	0.08	0.07	0.08	0.07

[1] 1 = 15 minutes before exercise
2 = immediately before exercise
3 = 5 minutes after exercise
4 = 20 minutes after exercise
5 = 60 minutes after exercise

In general the vitamin A levels increased following the two-step test. The carotene and tocopherol levels exhibited an average net decrease after an initial, transitory elevation. Considerable variation was observed in the plasma responses of all three substances in respect to time, direction and magnitude of the changes induced. Differences were also noted frequently between successive specimens before the exercise period.[2] Responses seemed uninfluenced by food ingestion before the test, and were no more consistent for the same than for different individuals.

[2] During the base-line period, the average change observed before exercise was in the same direction as the overall change after exercise for each substance, although the trend was temporarily reversed immediately following the step test, for both plasma carotene and tocopherol levels. There was no significant association between pre- and post-exercise changes in the same individual. In a few instances early morning plasma samples taken 2 to 4 hours before the test period showed higher concentrations of carotene and tocopherol than any subsequent specimens, suggesting a pre-test fall induced by the stress (physical and psychological) of travelling, and conceivably of anticipation. Limited control observations on two subjects further supported the concept of emotionally induced variation.

Observed changes in plasma concentrations are summarized and statistically evaluated in table 2.

In general, the major shifts in plasma concentration of vitamin A, carotene and tocopherols, notably average differences between overall pre- and post-exercise levels of both vitamin A and tocopherol, were statistically significant (P less than 0.05). The difference between comparable overall pre- and

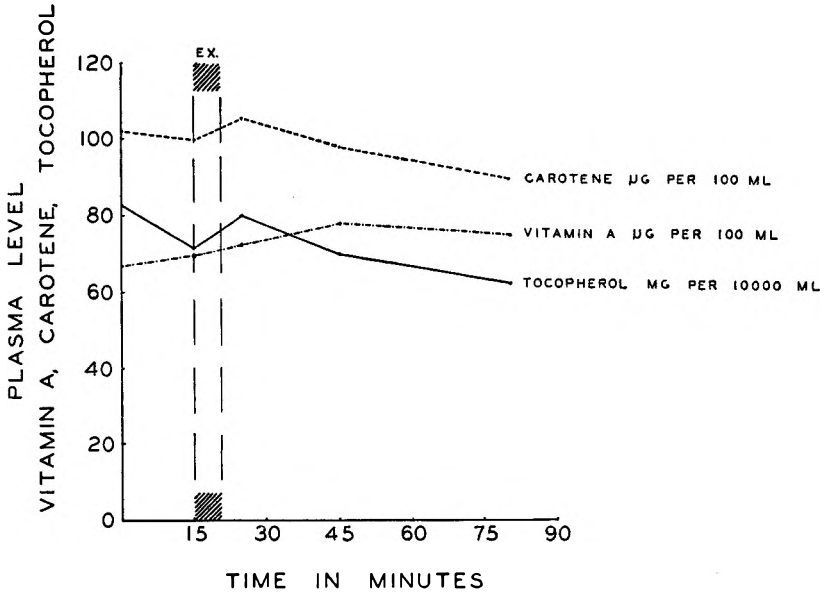

Fig. 1 Effect of exercise (Two-Step Test) on plasma concentration of vitamin A, carotene and tocopherol mean values, 30 tests, 14 subjects.

post-exercise averages for carotene was not significant. However, the final mean reading (one hour post-exercise) was significantly lower than the pre-exercise average concentration, and than the means of the second (pre-test) and third (5 minutes post-test) levels, respectively. The final tocopherol level also differed significantly from the mean pre-test level, the immediate pre-test level and the concentration 5 minutes after exercise.

TABLE 2

Mean plasma levels of vitamin A, carotene and tocopherols. Comparison and statistical evaluation of specimens before and after the two-step test

PLASMA COMPONENT	SPECIMEN COMPARISON [1]	MEAN	RANGE	SD	CV	SE	P VALUE	NUMBER OF CASES SHOWING			TOTAL NO. OF CASES [2]
								In-crease	De-crease	No Change	
Vitamin A, μg%	(1, 2) – (3, 4, 5)	+ 5.2	— 23 + 20	9.1	175	1.7	0.01	23	6	0	29
	1, 2) – (3)	+ 2.0	— 25 + 22	14.1	705	2.6	0.43	18	11	0	29
	(1, 2) – (5)	+ 4.9	— 33 + 60	18.2	371	3.4	0.16	18	9	1	28
	(3) – (5)	+ 2.2	— 54 + 49	22.3	1014	5.3	0.62	17	11	1	28
	(1) – (2)	+ 3.0	— 33 + 56	17.5	583	3.2	0.37	18	9	1	28
	(2) – (3)	+ 1.5	— 26 + 40	16.6	1107	3.2	0.62	12	15	1	28
	(2) – (5)	+ 3.8	— 35 + 42	15.4	405	2.9	0.19	18	10	0	28
Carotene, μg%	(1, 2) – (3, 4, 5)	— 2.7	— 24 + 16	10.3	381	1.9	0.19	16	11	2	29
	(1, 2) – (3)	+ 6.1	— 37 + 40	14.3	234	2.7	0.03	21	6	2	29
	(1, 2) – (5)	— 10.7	— 27 + 12	10.2	95	1.9	0.0001	27	2	0	29
	(3) – (5)	— 16.8	— 45 + 14	15.3	91	2.8	0.0001	3	25	1	29
	(1) – (2)	— 3.3	— 21 + 14	8.9	270	1.7	0.05	9	17	3	29
	(2) – (3)	+ 7.5	— 39 + 46	17.5	233	3.2	0.03	20	9	0	29
	(2) – (5)	— 8.6	— 36 + 17	11.0	128	2.0	0.0001	7	20	2	29
Tocopherols, mg%	(1, 2) – (3, 4, 5)	— 0.06	— 0.30 + 0.23	0.128	213	0.024	0.01	8	18	3	29
	(1, 2) – (3)	+ 0.02	— 0.25 + 0.30	0.134	670	0.025	0.45	13	12	4	29
	(1, 2) – (5)	— 0.16	— 0.55 + 0.20	0.189	118	0.035	0.0001	4	23	2	29
	(3) – (5)	— 0.15	— 0.70 + 0.80	0.256	171	0.047	0.01	3	20	6	29
	(1) – (2)	+ 0.05	— 0.60 + 0.60	0.207	412	0.038	0.22	14	6	9	29
	(2) – (3)	— 0.10	— 0.60 + 0.60	0.207	268	0.040	0.02	3	16	10	29
	(2) – (5)	— 0.11	— 0.50 + 0.30	0.496	178	0.036	0.01	5	19	5	29

[1] 1 = 15 minutes before exercise 2 = immediately before exercise 3 = 5 minutes after exercise 4 = 20 minutes after exercise 5 = 60 minutes after exercise. Discrepancies between these means and figures in table 1 reflect rounding off in calculation, do not affect statistical significance.

[2] Final (1 hour post exercise) specimen not available for one test.

The concordant association between the overall changes induced by exercise in the plasma tocopherol and carotene levels was statistically significant. (The correlation coefficient of 0.37 had a probability of 0.05.) The apparent discordant associations between changes in plasma vitamin A levels and those observed for carotene and tocopherol were not statistically significant.

There was no apparent correlation between the changes noted in the plasma vitamin levels and the physical condition of the subject as estimated subjectively and by the Brouha Index. The lack of association was especially evidenced in replicate trials in the same subjects, among whom the Brouha Index, but not the vitamin response, showed fair reproducibility.

DISCUSSION

The present findings agree only in part with those of James and his associates ('53). In general, as noted by these observers, the plasma vitamin A level was increased by exercise. In contrast to their final report (although consistent with their initial published impression), the average carotene level was significantly reduced. This apparent discrepancy doubtless results in part from wide variations observed in both studies; it is probably also related to the fact that the observations of James et al. were made soon after exercise (about 6 minutes) — a time when a temporary elevation in carotene content was also noted in the present series. Moreover, the two test situations were not comparable in terms of intensity or duration, nor were so-called training effects involved in the two-step test.

The apparent parallelism between changes in plasma carotene and tocopherol levels, and the seeming reciprocal association between these and the vitamin A response are not readily explained. The concentration of each of these substances exhibits a relationship to other components of the blood lipid fraction, although, except where impaired intestinal absorption is operative, their plasma levels show no con-

stant association. In general the evidence suggests that, where parallel changes in these moieties are not uniformly demonstrated, the carotene and tocopherol levels are more often positively associated than is either of these with the plasma A concentration (Urbach et al., '52; Van Bruggen and Straumfjord, '48).

The mechanisms governing these changes are obscure. The rapidity of occurrence suggests autonomic nerve, rather than, or at least in addition to, hormone effects — especially during the pre-exercise period of observation. Changes in plasma vitamin A and carotene levels have been induced by administration of epinephrine (Young and Wald, '40; Hillman, '49). Observations in this laboratory of subjects in the control state and following injections of this substance indicate probable effects of emotional stress on tocopherol levels as well, as have also been noted by Urbach et al. ('52). An attendant component of tension and anxiety cannot be dissociated in these tests from the impact of physical effort *per se*.

These observations indicate that, although physical exercise commonly induces significant changes in the plasma concentration of vitamin A, carotene and tocopherol, the variability and unpredictability of individual responses preclude their adaptation to the estimation of physical fitness — at least under conditions of this experiment. They further emphasize the need for considering the physical and emotional state, as well as dietary and related factors, in the interpretation of plasma levels of these substances.

SUMMARY

Plasma levels of vitamin A, carotene and tocopherol were determined before and following a two-step exercise test on 30 occasions in 14 subjects, including 14 tests in a single subject.

The mean vitamin A level increased after exercise; the mean carotene and tocopherol levels decreased after an initial transitory elevation. Considerable variation was observed in

respect to time, direction and magnitude of individual changes induced.

There was no apparent correlation between changes noted in the plasma levels and the physical condition of the subjects as estimated by the two-step test.

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IRON ABSORPTION AND METABOLISM

III. THE ENHANCEMENT OF IRON ABSORPTION IN RATS BY D-SORBITOL

JOHN F. HERNDON, EDWARD G. RICE, ROBERT G. TUCKER, EDWARD J. VAN LOON AND SAMUEL M. GREENBERG

Research and Development Division, Smith Kline and French Laboratories, Philadelphia, Pennsylvania

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In an earlier publication by Greenberg et al. ('57b) it was reported that D-sorbitol significantly enhances the absorption of vitamin B_{12} in rats. In a concurrent study Chow and coworkers ('58) reported that D-sorbitol also enhances the absorption of vitamin B_{12} in humans. Fournier et al. ('55) reveal that certain carbohydrates increase calcium absorption from the gut. Wasserman et al. ('56) found that several different amino acids, as well as lactose, would increase both calcium and strontium absorption in rats.

The current status and theories of the mechanisms of iron absorption have been reviewed by Gubler ('56). He pointed out that one of the chief factors controlling iron absorption is the amount of apoferritin in the gut wall, which appears to form only after iron is introduced into the gut lumen. He also stated that it is uncertain whether or not substances other than iron are involved in the stimulation of apoferritin formation. Groen et al. ('47) observed that certain substances, such as glutamic, aspartic, ascorbic, citric and tartaric acids, tend to enhance iron absorption and suggested that these substances did so by maintaining an acid pH which would prevent the precipitation of iron in the lumen of the gut. The beneficial effect of ascorbic acid on iron absorption in the rat has been reported by Greenberg et al. ('57a) and by Tucker and his associates ('57).

In view of previous findings that D-sorbitol enhances the absorption of vitamin B_{12}, a study was made of the influence of D-sorbitol on iron absorption in the rat.

MATERIALS AND METHODS

Young male and female Long-Evans strain rats were used in these studies. The results of 4 iron absorption experiments are reported here. Normal rats weighing approximately 250 gm, maintained on stock diet, were used in experiments I, II and III. Iron-deficient rats, weighing approximately 160 gm, made anemic by maintaining them on an iron-free diet described in an earlier publication (Tucker et al., '57), were used in experiment IV. In all experiments the groups of rats were balanced for litter weight and sex. All animals were fasted for a period of 18 hours prior to the initiation of each test. The conditions of each experiment were as follows:

Experiment I. Iron absorption in normal (non-anemic) rats. Eight rats were used: 4 rats (group 1) received by stomach tube 10 ml of test solution per kilogram body weight. Each 10 ml of the solution contained 2.5 mg Fe^{++} as $FeSO_4$ (carrier iron) and 5 μc of Fe^{59} in a 1% HCl solution. The remaining 4 rats (group 2) received an identical dose of radioactive Fe^{59} (and carrier iron) in a 28% D-sorbitol-1% HCl solution. The rats were refed one hour following the administration of the test solutions. The animals were housed in metabolism cages, and both feces and urine were collected daily for 4 days following intubation. The rats were sacrificed on the 4th day after 2.0 ml of cardiac blood had been removed. Following further exsanguination, the entire liver, spleen, and gastrointestinal tract with contents were removed and prepared for counting in a deep-well scintillation counter. Blood samples were counted directly; however, tissues were solubilized or digested and counts made on aliquots of these solutions. Total blood was calculated on the basis that blood represents 8% of total body weight (Cartland and Koch, '28).

Experiment II. Iron absorption in normal (non-anemic) rats. The conditions of this experiment were similar to those employed in the previous experiment. Whereas the test rats in experiment I were refed one hour after intubation, the rats in experiment II were refed 6 hours after intubation. In this and subsequent experiments, feces and urine were not collected. The levels of Fe^{59} in blood, liver, and spleen were measured in animals sacrificed on the 5th day post intubation. Blood samples were counted directly. Livers and spleens were solubilized and suitable aliquots were used for counting.

Experiment III. Effect of various levels of D-*sorbitol on Iron absorption in normal (non-anemic) rats.* Twenty-four normal rats were used to determine the effect of 6 different levels of D-sorbitol on iron absorption. Each rat received 10 ml of a 1% HCl solution containing 2.5 mg of Fe^{++} as $FeSO_4$ with 5 μc Fe^{59}/kg body weight. All rats received identical dosages of Fe^{59} and carrier iron. Group 1 received iron in water; groups 2, 3, 4, 5 and 6 received the same concentration of Fe^{59} and carrier iron in solutions containing respectively the following percentages of D-sorbitol: 1.75, 3.50, 7.00, 14.00, and 28.00. As in experiment II, the rats were refed the stock diet 6 hours after receiving the single intubations of the appropriate test solutions with iron. All animals in experiment III were sacrificed on the 5th day after intubation. Blood and liver levels of Fe^{59} were determined as in experiment II.

Experiment IV. Effect of various levels of D-*sorbitol on iron absorption in iron-deficient rats.* The experimental procedure duplicated that of experiment III except that iron-depleted rats were used instead of stock rats. Only blood was counted for radioactivity on the 5th day following the oral intubation of the test solutions of iron.

RESULTS

In experiment I, two different methods were employed for determining iron absorption: (1) measurements of tissue storage levels of radioactive iron and (2) determination of radio-

TABLE 1

Effect of D-*sorbitol on the absorption of* Fe^{59} *in stock rats (4 rats/group)*

GROUP NO.	TREATMENT ORAL INTUBATION [1] 10 ML/KG	% OF ORAL DOSE OF Fe^{59} [2]				
		Blood	Liver	Spleen	Gastrointestinal tract with contents	Excreta [3]
		Experiment I				
I	Fe^{59} (in 1% HCl)	2.5 ± 0.35	0.55 ± 0.06	0.03 ± 0.005	0.21 ± 0.03	96.8 ± 2.29
II	Fe^{59} (in 28% D-sorbitol-1% HCl solution)	4.7 ± 0.48	1.42 ± 0.51	0.07 ± 0.013	0.71 ± 0.13	83.4 ± 4.55
		Experiment II				
III	Fe^{59} (in 1% HCl)	1.7 ± 0.31	0.45 ± 0.72	0.03 ± 0.005		
IV	Fe^{59} (in 28% D-sorbitol-1% HCl solution)	7.8 ± 0.25	2.80 ± 0.55	0.12 ± 0.009		

[1] Each rat received 2.5 mg Fe++ carrier iron as $FeSO_4$ with 5 μc. Fe^{59}/kg.

[2] Includes standard error of the mean.

[3] Collected for 5 days following iron intubation; radioactivity measured on feces only.

active iron excreted. In subsequent experiments only the tissue storage technique was used. In experiment I (table 1) iron absorption, as measured by both techniques, was significantly greater in stock rats administered iron in a D-sorbitol solution than in those administered iron in water.

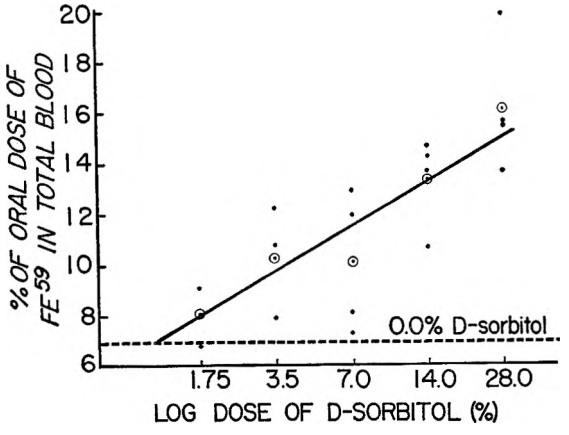

Fig. 1 Percentage of oral dose of Fe^{59} found in blood of normal rats 5 days after supplementation of iron (with Fe^{59}) in 10 ml D-sorbitol solution (0.0–28.0%) /kg body weight. Circled points represent average values at each level of D-sorbitol. Uncircled points represent individual values.

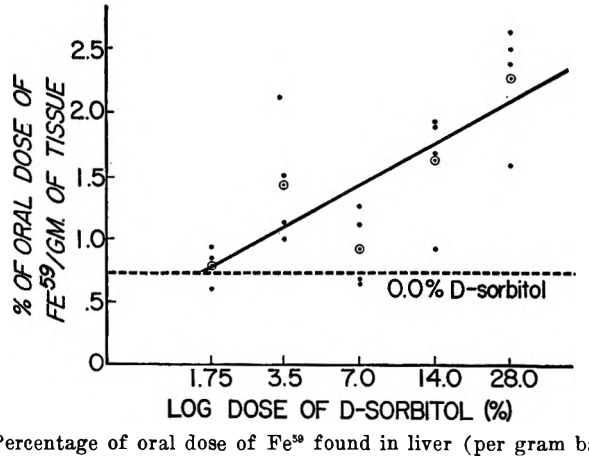

Fig. 2 Percentage of oral dose of Fe^{59} found in liver (per gram basis) of normal rats 5 days after supplementation of iron (with Fe^{59}) in 10 ml D-sorbitol solution (0.0–28.0%)/kg body weight. Circled points represent average values at each level of D-sorbitol. Uncircled points represent individual values.

The results of iron absorption studies in stock rats in experiment II (table 1) (tissue storage technique) substantiate the results observed in the first experiment.

The results of experiment III are presented in figures 1 and 2. Both blood levels and liver storage levels of Fe^{59} increased in proportion to the amount of D-sorbitol administered with the iron. The parallel increases in blood and liver storage levels of Fe^{59} in stock rats 5 days after oral intubation indicate that circulating radioactive iron represents a true reflection of iron absorption.

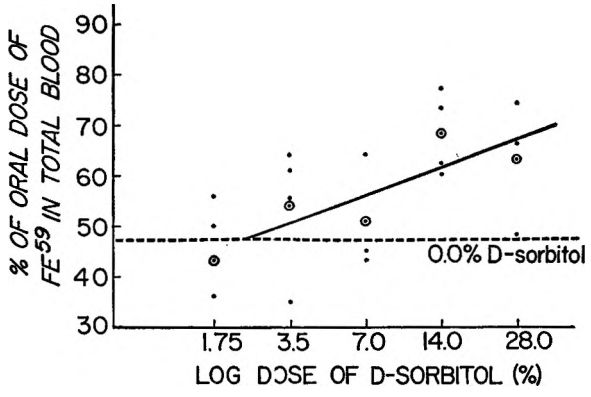

Fig. 3 Percentage of oral dose of Fe^{59} found in blood of anemic rats 5 days after supplementation of iron (with Fe^{59}) in 10 ml D-sorbitol solution (0.0–28%) /kg body weight. Circled points represent average values at each level of D-sorbitol. Uncircled points represent individual values.

The results of experiment IV are presented in figure 3 and show that iron-deficient rats respond to increasing levels of D-sorbitol in a manner similar to that observed in stock rats. Although the regression curves are similar, the response, expressed in percentage, was less marked in the deficient rats than in stock rats.

DISCUSSION

The importance of specific carbohydrates as activators or co-factors in the absorption mechanism is becoming apparent. Castle ('53) has suggested that the intrinsic factor might

function to facilitate the absorption of vitamin B_{12}, while Latner ('55) favors the concept that the intrinsic factor, a mucopolysaccharide, catalyzes B_{12} absorption. Greenberg et al. ('57b) postulate that certain carbohydrates either stimulate the absorption mechanisms of the intestinal wall or act by protecting various substances in the intestinal lumen.

Fournier and his collaborators ('55) showed that carbohydrates, which were active stimulators of calcium absorption, seemingly did not act through alterations of the intestinal flora and concluded that the enhancement of calcium absorption was a result of direct metabolic activity. Wasserman et al. ('56), who studied various amino acids and lactose for enhancement of calcium and strontium absorption, observed that the stimulant and the mineral had to be present in the gut simultaneously in order to obtain positive results. Furthermore, these workers suggest that the enhancing activity of the amino acids was not vitamin-like, since a relatively large ratio of amino acid to calcium was required. Our findings with iron absorption studies with D-sorbitol are in agreement with the observations of Wasserman et al.

In the previously reported work with vitamin B_{12} (Greenberg et al., '57b) and in the present work with iron, it was observed that above a certain critical level of D-sorbitol a direct relationship exists between the dosage of D-sorbitol administered and the level of absorption of these materials. Wasserman et al. ('56) reported progressive increases in calcium and strontium absorption with increased molar concentrations of lysine. Similar findings were observed in this laboratory when the effect of D-sorbitol concentration on vitamin B_{12} absorption was measured (unpublished data) and when the effect of D-sorbitol on iron absorption was measured (figs. 1–3).

Wissler and co-workers ('54) have tested the effect of 5% Tween 20 in the diet on iron absorption in the hamster for a period of 8 to 20 weeks. Tween 20, a fatty acid polyoxyethylene derivative of sorbitol, which also enhanced iron absorption, did not produce inflammation of the gastrointestinal mucosa.

Histological studies were made in our laboratory on the intestinal tracts of rats administered oral doses of 2.7 ml of 28% and 56% D-sorbitol solutions. The intestines were removed from the test rats one hour after the intubations and were found to be histologically normal when examined by our pathologist.

Increased absorption of iron usually has been associated with increased need for iron by an organism; however, D-sorbitol has caused an increased absorption of iron in nonanemic as well as in anemic rats. Wissler et al. ('54) also observed and reported that anemia was not an obligatory factor in Tween-stimulated iron absorption.

The results of these experiments indicate that substances other than iron are capable of playing a significant role in the iron absorption mechanism.

SUMMARY

D-Sorbitol is capable of enhancing significantly the absorption of iron in iron-depleted as well as in normal rats administered low levels of iron.

Above a critical minimal level of D-sorbitol, the absorption of iron is a direct function of the amount of D-sorbitol orally administered with the iron.

The possible mechanisms by which sorbitol and carbohydrate derivatives affect absorption are discussed.

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EFFECT OF AGE, SEX AND FEEDING REGIMEN ON FAT DIGESTIBILITY IN INDIVIDUAL RATS AS DETERMINED BY A RAPID EXTRACTION PROCEDURE

ROBERT L. SQUIBB,[1] ALVARO AGUIRRE,[2] J. EDGAR BRAHAM,[3] NEVIN S. SCRIMSHAW[3] AND EDWIN BRIDGFORTH[4]

Instituto de Nutrición de Centro América y Panamá (INCAP), Guatemala, C. A.

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Whenever rats have been used for determining the digestibility of fats, the experimental groups have been matched by age and sex and the resulting coefficients of digestibility usually reported on the basis of averages from the pooled feces of the whole group. In the course of earlier studies with rats on the nutritional value of several tropical fats, reported in part by Squibb et al. ('51) and Squibb and Fuentes ('52), it became apparent that age and sex differences were sources of variation.

During the present evaluation of the digestibility of two different aceituno fats, mature and young growing rats of both sexes were compared and the effects of the feeding

[1] Consultant in Animal Nutrition, Instituto de Nutrición de Centro América y Panamá (INCAP), Guatemala, C. A. Present address, Department of Poultry Science, Rutgers University.

[2] INCAP Fellow, assigned to the Instituto Agropecuario Nacional (IAN) and Servicio Cooperativo Interamericano de Agricultura (SCIDA), Guatemala, C. A.

[3] Director, Instituto de Nutrición de Centro Amércia y Panamá. A cooperative Institute for the study of human nutrition, supported by the Governments of Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama and administered by the Pan American Sanitary Bureau, Regional Office of the World Health Organization.

[4] Acting Chief, Divison of Statistics, INCAP, and Assistant Professor of Biostatistics, Vanderbilt University, School of Medicine. INCAP Scientific Publication I-46.

regimen and of variations between individuals in the digestion of fat were observed.

MATERIALS

The digestion trials were run in 1954, 1955 and 1956 on aceituno fats which were obtained from seeds of aceituno trees *(Simarouba glauca)* grown in El Salvador during the previous season.[5] Samples were obtained by (1) pressing whole seeds including the hulls and (2) pressing only hulled seeds. They were fed as both crude and partially refined fats. The refining process consisted of treating the fat with an amount of sodium hydroxide calculated to neutralize the free fatty acids and then heating it to the breaking point. The soaps were filtered off and the fat washed several times with water until free of alkali, and then dried.

METHODS AND RESULTS

Animal assay. For each of the experiments Wistar strain rats were maintained in individual all-wire cages with raised screen bottoms. The cages were housed in a room with a uniform temperature of approximately 72° F. Water was supplied ad libitum. For each of the experiments, rats of the same age were stratified by weight and then randomly assigned to the groups.

The fats were tested at a 15% level, replacing sucrose in the following low-fat basal ration: "vitamin-test" casein, 20 gm; sucrose, 76 gm; U.S.P. No. XII salt mixture, 4 gm; and the following vitamin supplements per 100 gm ration: Delsterol,[6] 20 mg; choline, 125 mg; thiamine, 0.2 mg; riboflavin, 0.35 mg; pantothenic acid, 1.2 mg; pyridoxine, 0.35 mg; and nicotinic acid, 1.5 mg.

Each fat was fed for a 5-day preliminary period followed by a 7-day collection period during which the feces of each individual rat were collected and stored under refrigeration

[5] Courtesy of H. de Sola e Hijos, San Salvador, El Salvador, C. A. For chemical data and physical characteristics of aceituno fat see Squibb et al. ('51).

[6] E. I. du Pont de Nemours and Company.

in all-glass containers until analyzed. When successive trials of fats were made using the same rats, a 7-day period was allowed between trials during which the regular stock colony ration was fed.

Determination of fecal fat. The fat content of the feces was determined by the following procedure which was developed to allow rapid extraction of 0.5 gm samples: feces were dried in an oven at 68°C for a period of 24 hours and then ground in a micro-Wiley mill to a mesh of approximately 60. Each fecal sample was analyzed in triplicate. A 0.5 gm sample was placed in a glass-stoppered test tube, treated with 5 drops of 18 N sulphuric acid, and then extracted with 10 ml of ethyl ether with constant shaking for 25 minutes. The tubes were centrifuged at 1800 rpm and the ether extract was transferred to individual weighing bottles. This extraction process was then repeated and the combined ether extracts dried in a current of warm air and subsequently in an oven at 60°C. Following drying they were cooled and weighed immediately.

As may be seen in figure 1, the reproducibility of this method is excellent. Only 6 out of 100 samples determined in triplicate had a coefficient of variation (ratio of standard deviation to mean value) over 4%, with 66 having less than 2%. Analysis of the data showed the percentage of fat in the feces to be independent of fecal volume. Digestion coefficients calculated from data obtained by this procedure gave slightly higher average results than those obtained with the more lengthy standard Soxhlet procedure (table 1) used by Augur et al. ('47).

Feeding trials. Experiments 1 and 2 were run simultaneously in 1954, using 5 adult male and three adult female rats of 8 months of age for each of three trials of ad libitum feeding of (1) crude aceituno fat, (2) a partially refined aceituno fat, and (3) refined cottonseed oil. The aceituno fat for experiment 1 was pressed from whole seeds and for experiment 2 from hulled seeds. Fat intake and excretion were determined for each rat.

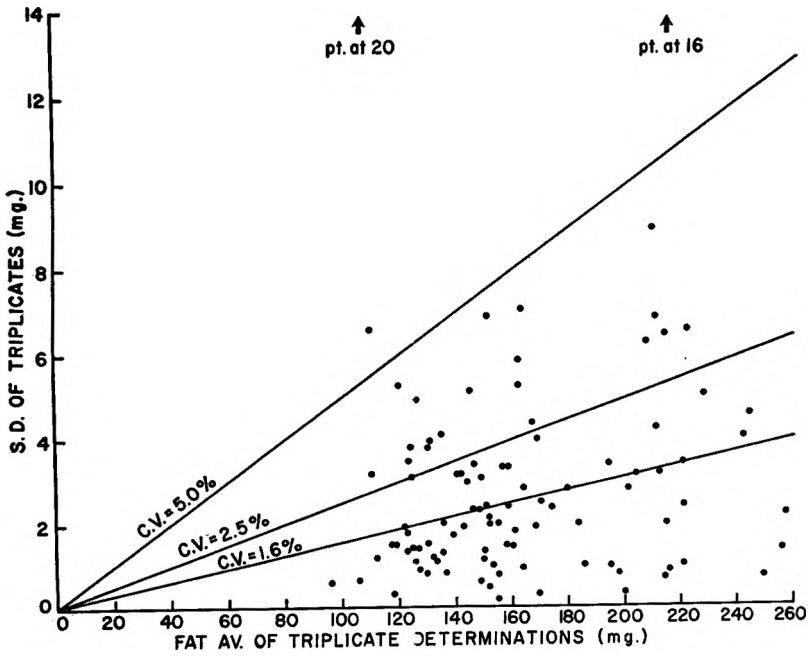

Fig. 1 Variation between triplicate replications of fecal fat determined on 100 samples by a rapid extraction procedure.

TABLE 1

Comparison of digestion coefficients (%) obtained from pooled rat fecal samples by a standard soxhlet fat extraction procedure and a semi-micromethod

(8 rats in each pooled sample)

POOLED SAMPLE	DIGESTION COEFFICIENTS		POOLED SAMPLE	DIGESTION COEFFICIENTS	
	Soxhlet [1]	Semi-micro [2]		Soxhlet [1]	Semi-micro [2]
1	89.8	90.0	8	89.1	88.8
2	92.7	92.3	9	93.0	92.6
3	85.5	88.6	10	90.9	91.4
4	92.9	92.9	11	88.0	89.4
5	88.3	90.1	12	85.1	85.6
6	90.0	90.5	13	94.4	94.3
7	85.2	87.0	14	89.2	89.4
			Average	89.6	90.2
			Difference	0.6 ± 0.3	

[1] Duplicate determinations.
[2] Triplicate determinations.

The digestion coefficients, presented in table 2, show that with ad libitum feeding the female adult rats digested all three fats significantly better than the males and with generally lower variability in response. The digestion of whole-seed aceituno fat was more efficient than digestion of hulled-seed

TABLE 2

Digestion coefficients (%) of aceituno whole-seed and hulled-seed fats[1] *(crude and partially refined) and cottonseed oil, fed ad libitum to mature rats*

(5 males and three females in each experiment)

MALES			FEMALES		
Trial 1 Crude aceituno fat	*Trial 2* Refined aceituno fat	*Trial 3* Refined cottonseed oil	*Trial 1* Crude aceituno fat	*Trial 2* Refined aceituno fat	*Trial 3* Refined cottonseed oil
Experiment 1. Aceituno whole-seed fat					
86.2	87.9	95.6	95.0	96.8	97.9
86.7	88.6	94.4	95.2	96.3	99.2
90.6	91.0	95.8	95.8	98.2	98.1
89.8	88.0	95.0			
87.0	86.3	92.9			
Mean 88.1	88.4	94.7	95.3	97.1	98.4
S.D. 2.0	1.7	1.2	0.4	1.0	0.7
Experiment 2. Aceituno hulled-seed fat					
82.7	89.0	96.7	93.1	93.6	99.3
85.3	81.9	94.6	87.2	93.3	98.0
77.2	83.9	95.3	88.5	93.0	97.8
86.6	88.0	94.8			
81.6	85.2	97.3			
Mean 82.7	85.6	95.7	89.6	93.3	98.4
S.D. 3.7	2.9	1.2	3.1	0.3	0.8

[1] Harvested in 1953.

fat. The rats given whole-seed fat averaged a non-significant rise of $0.8 \pm 0.5\%$ in fat digestion when changed from crude to partially refined fats. Those given hulled-seed fat averaged a significant rise of $3.2 \pm 1.2\%$ when changed from crude to partially refined fat, however, but did not rise to as high a level of utilization as the rats receiving the refined whole-seed product. None of the aceituno samples, whether crude or re-

fined, were digested as efficiently as cottonseed oil, for which digestion coefficients were 95% for males and 98% for females.

Experiments 3 and 4 were run simultaneously in 1955 to determine if the sex differences in fat digestibility observed in adult rats of experiments 1 and 2 would be apparent in young growing rats, and to investigate the effect of moderate restriction of intake. In each experiment, 8 male and 8 female rats 6 weeks of age were used for two trials: (1) ad libitum feeding, and (2) restricted feeding, limiting each rat to the same intake as that of the lowest ad libitum intake of a female rat. This was approximately 85% of the average ad libitum intake of females and 72% of that of the males.

The partially refined aceituno fat (experiment 3) was obtained from whole seeds and the unrefined fat (experiment 4) was obtained from hulled seeds. In these young growing rats there was no indication of a sex difference in digestibility for either fat or feeding regimen (table 3). In experiment 4 there was no indication of a change in utilization with restriction of intake, but in experiment 3 the use of refined aceituno whole-seed fat resulted in an average reduction in digestibility for both males and females ($2.1 \pm 0.8\%$ for females, $1.4 \pm 0.7\%$ for males). The variability of individual differences in changing utilization between the two trials was similar for both experiments and both sexes; the standard deviation was 2%.

Experiment 5 was performed to test the effect of moderate restriction of intake in mature rats, using 16 adult males and 16 adult females 7 months of age for two trials: (1) ad libitum feeding, and (2) restricted feeding, limiting each rat to the lowest ad libitum intake of the females, which was approximately 90% of the average ad libitum intake of females and 80% of that of males. The aceituno fat, as in experiment 4, was unrefined but from hulled seeds.

The higher fat utilization by females previously noted for mature rats on ad libitum intakes was again evident (table 4). On restricted intake the sex difference was not significant ($89.8 \pm 0.8\%$ for females, $88.4 \pm 0.5\%$ for males). Further,

both sexes showed changes from their ad libitum intake utilizations which were not, however, of statistical significance: females fell by $1.4 \pm 0.7\%$ ($P = .09$), and males rose by $1.7 \pm 0.9\%$ ($P = .08$).

Analysis of the data of experiments 3, 4 and 5 showed that for ad libitum and restricted feeding the correlations between digestion coefficients were 0.80, 0.72 and 0.48 for males and 0.70, 0.01 and 0.01 for females.

TABLE 3

Digestion coefficients (%) of refined aceituno whole-seed fat and crude aceituno hulled-seed fat,[1] fed ad libitum and with moderate restriction to growing rats

(8 males and 8 females in each experiment)

MALES		FEMALES	
Trial 1 Intake ad libitum	*Trial 2* Intake restricted	*Trial 1* Intake ad libitum	*Trial 2* Intake restricted
Experiment 3. Refined aceituno whole-seed fat			
94.7	93.3	91.1	89.7
94.7	91.6	92.3	92.4
94.6	97.1	95.4	93.5
95.0	93.7	95.6	94.4
89.3	89.7	94.1	94.6
89.9	86.1	95.8	94.4
91.9	89.9	97.0	90.8
92.2	89.8	90.1	84.9
Mean 92.8	91.4	93.9	91.8
S.D. 2.3	3.3	2.5	3.3
Experiment 4. Crude aceituno hulled-seed fat			
91.8	91.0	88.8	89.2
89.4	89.6	90.7	89.2
92.1	89.8	88.4	90.6
92.3	92.2	90.6	90.8
87.0	86.5	90.3	90.2
89.4	88.1	90.1	88.1
84.7	88.7	89.3	90.9
88.8	89.2	88.5	86.7
Mean 89.4	89.4	89.6	89.2
S.D. 2.7	1.7	1.0	1.7

[1] Harvested in 1954.

TABLE 4

Digestion coefficients (%) of crude ceituno hulled-seed fat,[1] fed ad libitum and with moderate restriction to mature rats

(16 males and 16 females)

MALES		FEMALES	
Trial 1 Intake ad libitum	*Trial 2* Intake restricted	*Trial 1* Intake ad libitum	*Trial 2* Intake restricted
	Experiment 5		
88.1	91.4	92.2	94.2
85.3	90.1	90.3	91.2
87.0	81.1	89.7	91.5
83.6	89.5	90.2	89.8
88.4	91.4	90.6	93.8
79.7	88.5	92.1	93.0
87.4	90.8	94.1	94.3
87.4	88.1	90.9	89.2
89.2	86.6	90.8	84.9
90.3	88.3	89.3	89.0
87.0	88.5	92.2	90.5
88.3	85.2	87.4	84.0
86.5	85.8	92.1	87.5
87.9	87.9	93.2	88.1
89.6	87.5	92.6	90.9
88.2	88.2	90.7	84.9
Mean 86.8	88.4	91.2	89.8
S.D. 3.0	1.8	1.7	3.3

[1] Harvested in 1956.

DISCUSSION

In biological research, samples are pooled for analysis for reasons of time and economy. The chemical procedure suggested here may be carried out in triplicate on individual samples with excellent reproducibility and with a considerable saving in time over Soxhlet fat extraction procedures. This method has not been used for the extraction of the feces of rats fed highly saturated fats. While only total fat is obtained by the procedure described, it is possible to extract feces first without acidfying and thus separate the neutral fat and fatty acids.

The kind and state of a fat has a definite relation to the degree of digestibility. Partial refining of the crude aceituno fats not only increased digestibility but also lessened individual variation.

The requirements of rats of different age and sex for maintenance, growth and reproduction influence utilization of fat. With the exception of trial 2, experiment 5, the mature females digested fat with less variation than mature males. This sex difference was not apparent in the young growing rats. Further, mature females fed an ad libitum regimen digested fat significantly better than did the males. Again, this effect of sex was not apparent when the fat intake of the mature males was restricted to that of the female or young growing rats. In order to understand these phenomena better, series of calculations were made from the observed data. These showed, first, that according to the type of fat fed and the feeding regimen employed, there was a significant difference in percentage of fat in the feces of mature males and females when fed the same fat ad libitum. For mature males the values ranged from 4.3 to 17.3% and for females from 1.6 to 10.4%. However, when the diet was restricted there was no significant difference between the sexes in the percentage of fecal fat. This was also true of young growing rats; however, in this case it was irrespective of feeding regimen. Second, calculations of the fat intake per unit of body weight showed that young female and male rats consumed an equal amount of fat per day per gram of body weight when fed ad libitum (0.0065 vs. .0064 gm). On a restricted regimen the males consumed significantly less than the females (0.0038 vs. 0.0065 gm), yet the coefficients of digestibility under both regimes remained the same. When adult rats, on the other hand, were fed according to either a restricted or an ad libitum regimen, the females consumed significantly more fat per day per gram of body weight than the males (0.0066 vs. 0.0047 gm); only in the case of ad libitum feeding were the coefficients of digestibility significantly greater for females than for males.

Although the sample size was relatively small, this investigation has disclosed that procedures for the determination of the digestibility of fat with rats may in some cases be subject to considerable error if they fail to take into account not only the type of fat but also the sex, age and feeding regimen. A careful standardization of procedures is necessary to help to control these variables.

SUMMARY

A rapid procedure for the extraction of the fecal fat of rats used in digestion trials is presented. Data obtained by this method demonstrate a marked variation among individual rats of both sexes in the digestion of fat. This variation appears to be greater in adult males; however, restricting the fat intake of the adult male to that of the female lessened the variation. When fed ad libitum, mature females digested more fat than males. This sex difference was not apparent when the fat intake of the males was restricted to that of the females or when young growing rats of either sex were kept on ad libitum or restricted feeding regimens. Since variations in utilization were found to occur with the type of fat, sex and age of the rats and feeding regimen, the importance of standardizing procedures for determining fat digestion coefficients with rats is indicated.

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FATTY LIVER OF PORTAL TYPE: CURED BY LYSINE PLUS TRYPTOPHAN

GEORGE P. VENNART, VINCENT P. PERNA [1] AND WELLINGTON B. STEWART

Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, N. Y.

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Previous experiments have shown that fat accumulates in the periphery of liver lobules if weanling rats are fed a diet in which corn forms the only source of protein (Shils et al., '54). It was further shown that adding lysine plus tryptophan to the diet prevented the fatty changes (Shils and Stewart, '54b), while methionine, choline, and vitamin B_{12} were ineffective (Shils et al., '55). It is naturally of interest to determine whether the fatty change, once induced, is reversible by agents which prevent the lesion. In general, it can be expected that such will be the case, but there remains the possibility that the lesion is irreversible or that other substances may be required to induce a cure.

The experiments reported here show that the type of fatty liver produced by corn diets is reversed by the addition of lysine plus tryptophan.

METHODS

The rats were of the same strain (Shils and Stewart, '54a) used in previous experiments. At weaning (21 days) the young rats were placed on diet 90 composed of: corn meal 78, salts [2] 3, vitaminized cerelose-1 (Shils and Stewart, '54a), 3, 10% cod liver oil in corn oil 1 and hydrogenated [3] vegetable

[1] Present address: Department of Pathology, University of Missouri, Columbia.

[2] Hubbell, R. B., L. B. Mendel and A. J. Wakeman. J. Nutrition, *14:* 273, 1937.

[3] Crisco.

oil 15%. To each 100 gm of this diet was added choline, 263 mg; vitamin E, 26.3 mg; vitamin K, 26.3 mg, and vitamin B_{12}, 105 μg. This diet differs from those previously used in this laboratory by the addition of these supplements. The portal distribution of liver lipid remained unchanged by this modification. At the end of 14 to 28 days the animals were divided into three groups in accordance with litter-mate-pair and sex distribution techniques. Those of group I were killed by exsanguination under pentobarbital anesthesia. At 28 days a second third of the animals (group II) was supplemented with 0.5% of DL-tryptophan and 1.0% of L-lysine (diet 91) and maintained on this diet for an additional 28 days. The final third (group III) of the animals remained on the unsupplemented diet. At the end of the experimental period both groups II and III were killed. Complete autopsies were performed and the livers treated as described previously (Shils et al., '54b). Lipids are expressed on a wet weight basis.

RESULTS

As is shown in the table, the basic diet caused an elevation of liver lipids above the normal of 2 to 3%. No significant difference in lipid levels could be demonstrated between groups I and II. The rats of group III, supplemented with lysine plus tryptophan, had a normal level of liver lipids after 28 days. It should be pointed out that the poor growth of the animals was not improved by the addition of lysine plus tryptophan.

TABLE 1

The effect of lysine plus tryptophan on liver lipids

GROUP NO.	NO. OF RATS	TIME ON DIET 90	TIME ON DIET 91	FAT IN LIVER	AV. WT. GAIN
		weeks	*weeks*	%	*gm*
I	47	4	0	6.63 ± 0.410 [1]	6.0
II	40	8	0	7.33 ± 0.630	11.4
III	34	4	4	2.76 ± 0.142	11.1

[1] Standard deviation.

The results of the microscopic examination of the livers supported the chemical data. In groups I and II the stainable lipid (Sudan IV) had a clear-cut portal distribution. The lipid granules were uniformly small except for a rare cell with a large vacuole. The livers of rats supplemented with lysine plus tryptophan contained little or no stainable lipid.

DISCUSSION

The results of these experiments indicate that portal fatty metamorphosis induced by our basic diet is completely reversed by the addition of lysine plus tryptophan. In no instance was there any demonstrable permanent damage. This is in keeping with the concept that fatty metamorphosis represents a variable but usually mild degree of cellular damage and that the removal of the cause results in a return to normal of structure and function.

The term "amino-acid imbalance" has been used to describe many functional and anatomical changes induced by diets having poor ratios of amino acids. However, the use of this term does not illuminate the processes underlying the production of the lesions. In the current instance, the mechanism by which the fat is caused to accumulate in the liver cells remains obscure. These experiments and those reported earlier by us and others suggest a possible relationship between protein and lipid metabolism that has not yet been elucidated.

SUMMARY

Portal fatty metamorphosis in young rats subsisting on diets in which protein is derived solely from corn is reversed by the addition of tryptophan plus lysine to the diet.

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