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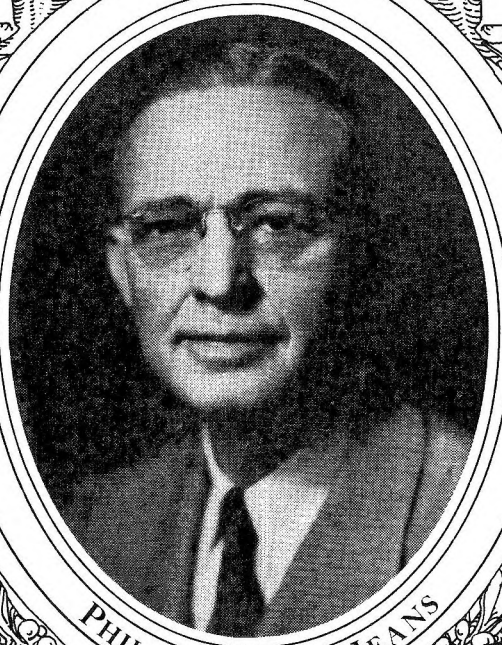

PHILIP CHARLES JEANS

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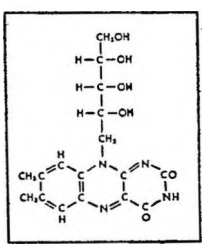
The Vital Story

A Quick History. Independent investigators, working separately to unlock several of nature's doors, sometimes open up unsuspected relationships. This happened with vitamin B_2.

Investigations. About 25 years ago, several groups, notably Warburg's, were investigating a "yellow enzyme" obtained from yeast. Almost simultaneously other investigators were studying a food factor that aided growth of laboratory animals.

What they found. Proceeding with chemical analysis of this growth factor, the team of Kuhn, György, and Wagner-Jauregg noted a relationship between the growth-producing agent and the "yellow enzyme." Their findings, and those of other researchers along similar lines, were published in 1933. Eventually, riboflavin and an essential part of the yellow enzyme were found to be identical and the unity of an essential nutrient and cellular metabolism was established.



Isolation of pure riboflavin was achieved by Kuhn and his co-workers, and by Ellinger and Koschara, in 1933.

Nomenclature. Known in the United States as riboflavin, this vitamin has also been called lactoflavin, ovoflavin, hepatoflavin, and vitamin G.

SYNTHESIS

By 1935, two eminent chemists, working separately, had synthesized riboflavin, practically in a dead heat. Prof. Paul Karrer of the University of Zurich, a collaborator of the Hoffmann-La Roche Laboratories, produced the first successful synthesis. Five weeks later Richard Kuhn of Germany announced his synthesis of the vitamin. Prof. Karrer subsequently shared the Nobel Prize in Chemistry for his work in vitamins and carotenoids.

The Karrer synthesis forms the basis for chemical processes in widespread use today by Hoffmann-La Roche and other leading manufacturers throughout the world. Riboflavin is also manufactured today by fermentation methods.

CHEMICAL AND PHYSICAL PROPERTIES

Riboflavin is yellow, slightly water-soluble with a greenish fluorescence and a bitter taste. Its empirical formula is $C_{17}H_{20}N_4O_6$. Vitamin B_2 produced by the Roche process is identical in every way with that occurring in nature.

How does vitamin B_2 work? Riboflavin is a vital part of nature's chain of reactions for utilization of carbohydrate energy. It has been found to be a constituent of many enzym systems and is thus intimately connected with life processes It is probably required by the metabolic processes of every animal and bird as well as by many fishes, insects and lower forms of life. (In certain animals, however, the requirement may be synthesized by bacteria within the intestine.)

In the cells riboflavin goes to work attached to a phosphate group. This substance, known as riboflavin-5′-phosphate or flavin mononucleotide, may in turn be attached tc still another essential substance, adenylic acid, forming flavit adenine dinucleotide. Either nucleotide then is attached tc protein, thereby forming an enzyme, and takes its part ir oxidation-reduction reactions.

Requirements in Human Nutrition. As we have seen, vita min B_2 is essential to life. We have no special storage organs in our bodies for this vitamin, although a certain level i: maintained in various tissues, with relatively large amount found in the liver and kidneys.

MEASURING METHODS

In the beginning, riboflavin activity was described in "Bour quin-Sherman units" and requirements were thought to be very small. Subsequent research showec otherwise. Milligrams of weight becam the unit and the Food & Drug Adminis tration of the U. S. Dept. of Health Education & Welfare has established minimum daily requirement of 2.0 mg of riboflavin for all persons 12 or more years old. For infant it is 0.5 mg. These requirements are designed to prevent the occurrence of symptoms of riboflavin deficiency disease. The minimum daily requirement for this vitamin for children from 1 to 12 years has not been established by the F. & D. A

Recommended allowances. The Food & Nutrition Boar of the National Research Council has recommended the fol lowing daily dietary allowances of riboflavin, expressed as milligrams. These are designed to maintain good nutrition of healthy persons in the U. S. A.

			Boys	Girls
Men			1.6	
Women			1.4	
"	(3rd trimester of pregnancy)		2.0	
"	(Lactating)		2.5	
Infants,	1-3 months		0.4	
"	4-9 "		0.7	
"	10-12 "		0.9	
Children,	1-3 years		1.0	
"	4-6 "		1.2	
"	7-9 "		1.5	
Adolescents,	10-12 years		1.8	1.8
"	13-15 "		2.1	2.0
"	16-20 "		2.5	1.9

# of VITAMIN B_2 *by* Science Writer

(Riboflavin)

ficiencies of vitamin B_2 appear in several ways in human ngs. The eyes, the skin, the nerves, and the blood show the ects of too little riboflavin. Laboratory mals have demonstrated that a ribo- vin-deficient diet can cause death of ılts and can slow or stop growth in the ıng. Female animals, deprived of ribo- vin in the diet, may produce offspring h congenital malformations.

ıdical uses. To overcome and control deficiencies in ıan beings, physicians have pure riboflavin available for ıinistration by injection or orally, by itself or with other " vitamins or multi-vitamin-mineral combinations.

w do we get our daily riboflavin? Vitamin B_2 has ıe distribution throughout the entire animal and vegetable gdoms. Good sources are milk and its products, eggs, ats, legumes, green leaves and buds. Whole-grain cereals ve significant but not large amounts of riboflavin.

ADDITION TO FOODS

Cereal foods play a large part in our diet. To produce the white flour almost all of us want, millers are obliged to remove parts of the wheat that contain much of the grain's riboflavin and other nutrients. In addition, cereal grains are not rich sources of riboflavin. Millers meet this problem by *-iching* the grain foods for which federal standards exist h vitamins B_1, B_2, niacin and the mineral iron. In the case vitamin B_2, however, they do more than *restore* the proced food to its natural riboflavin level; they *fortify* the food h enough of this essential vitamin to make it nutritionally re valuable than it was in nature.

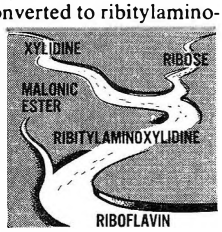
ting to protect the good health of millions of Americans, ers and millers adopted *enrichment* of white bread and te flour in 1941. Since that time, r foods, such as macaroni prod- , corn meal and grits, farina, tina and breakfast cereals have their food value increased by chment with pure riboflavin other vitamins and minerals.

en enriching, fortifying or restoring, food manufacers add the necessary quantity of riboflavin (and other mins and minerals) to the food during processing, so that finished product meets federal, state, and territorial rements or contributes to the consumer an amount of the min that dietary experts believe significantly useful.

PRODUCTION

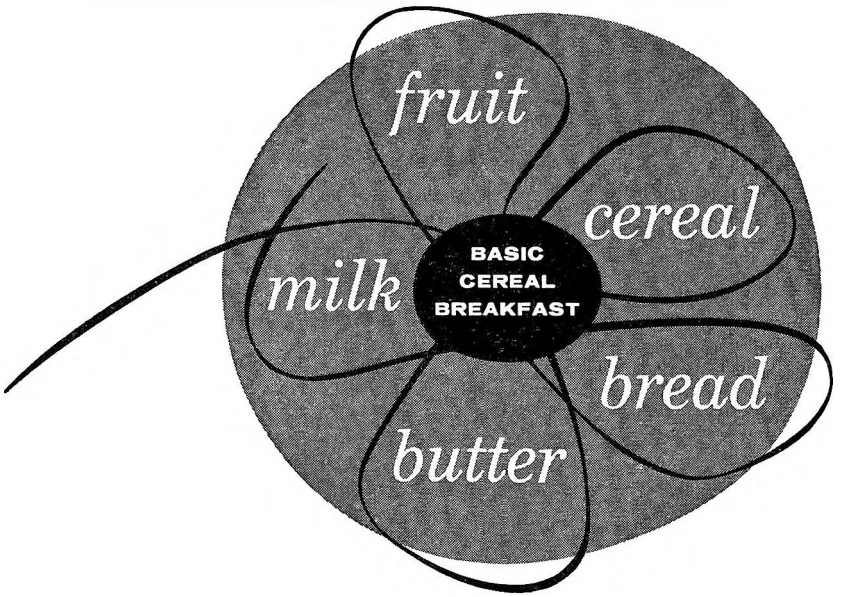
f. Karrer's synthesis of riboflavin was a laboratory suc- Adapting the process to commercial production, however, demanded original thinking by chemists at Hoffmann-La Roche. The production of riboflavin by chemical synthesis requires the production of ribose, a rare sugar, at an early stage in the process. This special sugar must be made inexpensively if the synthesis is to be practical. Sugar chemistry is a difficult matter. In a brilliant piece of work, the Roche chemical experts developed a method to produce ribose on a commercial scale by an electrolytic process, thus overcoming a most troublesome problem. Subsequently, Roche chemists developed the first practical synthesis for riboflavin-5′-phosphate, identical with natural flavin mononucleotide.

Picture three streams joining to form a river and you have a simplified idea of the Roche process for synthesizing vitamin B_2. O-xylene and glucose are processed separately to form xylidine and ribose respectively. These are joined to form ribitylxylidine, which is then converted to ribitylaminoxylidine. Starting separately with malonic ester, which is processed through intermediate stages to alloxan, the third "stream" is then joined with ribitylaminoxylidine to form riboflavin. Purification occurs at each step of the synthesis. Riboflavin 'Roche' equals or exceeds U. S. P. standards.



By the tons. So efficient is the Roche process that pure riboflavin is produced *by the tons* for use in pharmaceutical products and processed foods. An interesting development by Roche is the production of riboflavin in different forms related to the method of end use. 'Roche' Regular riboflavin U. S. P. is especially useful in dry enrichment premixes, powdered dietary supplements, pharmaceutical tablets and soft gelatin capsules. 'Roche' Solutions type is preferred for the manufacture of solutions having low concentration. 'Roche' Riboflavin-5′-Phosphate Sodium is a highly and rapidly soluble riboflavin compound favored for all pharmaceutical liquid products and some tablets, lozenges, and capsules. It has a more pleasant taste than the bitter U. S. P. riboflavin.

This article is published in the interests of pharmaceutical manufacturers, and of food processors who make their good foods better using pure riboflavin 'Roche.' Reprints of this and others in the series will be supplied on request without charge. Also available without cost is a brochure describing the enrichment or fortification of cereal grain products with essential vitamins and minerals. These articles and the brochure have been found most helpful as sources of accurate information in brief form. Teachers especially find them useful in education. Regardless of your occupation, feel free to write for them. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 1956 Bourdon St., St. Laurent, P. Q.



are you interested in a *low-fat* quick and lasting energy breakfast?

When a reduction of fat in the diet is indicated in the morning meal, the fat calories can be replaced by those of *low-fat content* yet providing well-balanced nourishment and quick and lasting energy throughout the morning hours.

The Iowa Breakfast Blood Sugar Studies proved that a basic cereal and milk breakfast providing about 20 gm. mixed plant and animal protein (cereal and milk) provided quick and lasting energy throughout the early and late morning hours. As shown in the table below this morning meal is *low in fat* and provides well-balanced nourishment.

basic cereal low-fat breakfast pattern

Orange juice, fresh, ½ cup,
Cereal, dry weight, 1 oz.,
with whole milk, ½ cup, and sugar, 1 tsp.,
Bread, white, 2 slices, with butter, 1 tsp.,
Milk, nonfat (skim), 1 cup,
black coffee

Nutritive value of basic cereal breakfast pattern

CALORIES	502	VITAMIN A	600 I.U.
PROTEIN	20.5 gm.	THIAMINE	0.46 mg.
FAT	11.6 gm.	RIBOFLAVIN	0.80 mg.
CARBOHYDRATE	80.7 gm.	NIACIN	3.0 mg.
CALCIUM	0.532 gm.	ASCORBIC ACID	65.5 mg.
IRON	2.7 mg.	CHOLESTEROL	32.9 mg.

Note: To further reduce fat and cholesterol use skim milk on cereal which reduces Fat Total to 7.0 gm. and Cholesterol Total to 16.8 mg. Preserves or honey as spread further reduces Fat and Cholesterol.

Bowes, A. deP., and Church, C. F.: Food Values of Portions Commonly Used. 8th ed. Philadelphia: A. deP. Bowes, 1956.
Cereal Institute, Inc.: The Nutritional Contribution of Breakfast Cereals. Chicago: Cereal Institute, Inc., 1956.
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A CRITICAL EVALUATION OF *MYO*-INOSITOL AS AN ASCORBIC ACID-SPARING AGENT [1]

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(Received for publication August 2, 1957)

One of the more interesting nutritional claims which has been made for *myo*-inositol [2] is that it is an effective agent for controlling scurvy in guinea pigs. Thus, De Felice ('50, '53) reported that it prevented the neuromuscular symptoms of scurvy and retarded the loss of ascorbic acid from the organs of guinea pigs on a scorbutigenic diet. And Oggioni ('53) claimed nearly complete prevention of scurvy in guinea pigs by administration of inositol, and nearly complete cures for those animals already suffering from scurvy.

In the rat, ascorbic acid is synthesized from glucose, and possibly galactose, (Mapson, '54) and inositol seems to be converted to some extent to glucose (Stetten and Stetten, '46; Posternak, Schopfer and Reymond, '55). It seemed possible, therefore, that there might be intermediates in these two metabolic pathways which are slowly interconvertible, and which if available in sufficient quantities, could be used by the guinea pig for the synthesis of ascorbic acid even though the animal has lost its ability to synthesize this vitamin directly from glucose. The results of some experiments on the effect of inositol on ascorbic acid synthesis in plants (Banerjee and Nandi, '49; Devyatnin, '50) lent support to this idea.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and in part by a grant from the National Vitamin Foundation.

[2] Also called *meso*-inositol, *m*-inositol, *i*-inositol, and inositol.

In view of the interesting implications of the claims of De Felice and Oggioni, it was decided to see whether these claims could be confirmed.

EXPERIMENTAL AND RESULTS

Experiment 1 was a preliminary experiment. Male guinea pigs [3] weighing 500 to 600 gm each were divided into groups of 6. The basal diet,[4] which was fed ad libitum, contained dextrin, 25% and sucrose, 16% as carbohydrates. It was otherwise essentially the same as the diet used in experiment 3, below. The animals were supplied weekly with vitamins A, D, E, and K. Supplements of inositol and ascorbic acid, when given, were administered daily by pipet, the inositol as a 10% aqueous solution and the ascorbic acid as a 1% solution in 20% sucrose. There were three control groups: I, basal diet + ascorbic acid; II, basal diet only; and IV, basal diet + ascorbic acid + inositol (from the beginning); and two inositol test groups: III, basal diet till scorbutic, then + inositol; and V, basal diet + inositol from the beginning.

Although two of the animals died of a respiratory infection, none of the guinea pigs in the groups receiving ascorbic acid (I and IV) showed any symptoms of scurvy during the experimental period. Three animals belonging to the other groups died early in the period of the respiratory infection; the remainder began to show evidence of scurvy (sore joints, "face ache" position, bleeding gums, diarrhea and weight loss in various combinations) during the third week and died shortly thereafter. There was no evidence that inositol, administered either from the beginning or after symptoms developed, had any effect on the course of the scurvy.

Experiment 2. It seemed possible that the demonstration of an antiscorbutic effect of *myo*-inositol depended on some unknown substance present in the natural diet used by Oggioni, but not in the purified diet used in experiment 1. An attempt was therefore made to duplicate Oggioni's experi-

[3] Obtained from Gopher State Caviary, St. Paul, Minnesota.
[4] Dr. H. R. Heinicke, personal communication.

ment as closely as possible. The basal diet consisted of crimped oats, sun-cured alfalfa hay, and water, each fed ad libitum in a separate container. Male guinea pigs from the same commercial source were again used, 6 per group. The weights ranged from 400 to 700 gm. When supplements of inositol and ascorbic acid were given, they were again administered by pipet, at the following levels: inositol, 10 ml of 10% solution per animal per day; ascorbic acid, 2 ml of 1.25% solution in 20% sucrose per animal per day.

The regimens used, and the results obtained, are shown in table 1. It is clear that, when administered either from the start or after scurvy had set in, inositol had no effect on mortality in the ascorbic acid-deficient groups. The animals of the inositol-supplemented groups showed slightly less weight loss during the last few days of the experiment, but the differences from the control group are of questionable significance.

Gross observations indicated that scurvy was the cause of all the deaths which occurred. There was no evidence that the scorbutic symptoms were in any way alleviated by the administration of inositol.

Experiment 3. The negative results of the first two experiments were obtained with diets essentially devoid of ascorbic acid. It seemed possible, however, that in a diet containing suboptimal amounts of this vitamin, *myo*-inositol might spare ascorbic acid, and it seemed likely that, if such a sparing action were to be observed, it would be observed through its effect on growth. Young guinea pigs were therefore used, and a purified diet which gives reasonable growth when supplemented with adequate vitamin C was chosen.

The basal diet (Collins and Elvehjem, '58) contained alcohol extracted casein, 30%; sucrose, 40%; corn oil, 7.4%; cellulose flour,[5] 15%; salts IV (Hegsted et al., '41), 4%; potassium acetate, 2.5%; magnesium oxide, 0.5%; choline, 0.35%; and vitamin mix, 0.25%. The vitamin mix included the following,

[5] Solka-Floc BW–200, manufactured by the Brown Co., Berlin, New Hampshire.

TABLE 1

Experiment 2

The effect of myo-*inositol and ascorbic acid on the weights and survival of adult guinea pigs fed a scorbutigenic diet of hay and oats*

DAYS ON EXPERIMENT	AVERAGE WEIGHTS				
	Group I No supplement	Group II + Inositol	Group III + Inositol after onset of scurvy [1]	Group IV + Ascorbic acid after onset of scurvy [1]	Group V + Ascorbic acid
	gm	*gm*	*gm*	*gm*	*gm*
10	488 ± 42 [2] (6) [3]	479 ± 37 (6)	478 ± 19 (6)	503 ± 41 (6)	500 ± 44 (6)
20	391 ± 41 (6)	414 ± 33 (5)	385 ± 24 (6)	418 ± 40 (6)	560 ± 42 (6)
24	320 ± 38 (6)	348 ± 28 (5)	319 ± 19 (6)	429 ± 43 (6)	567 ± 40 (6)
26	270 ± 13 (4)	315 ± 29 (5)	311 ± 13 (4)	433 ± 43 (6)	577 ± 37 (6)
28	230 ± 10 (4)	259 ± 14 (3)	281 ± 15 (3)	443 ± 44 (6)	595 ± 39 (6)
30	— (0)	242 ± 2 (2)	255 ± 9 (2)	460 ± 45 (6)	595 ± 35 (6)
33	— (0)	— (0)	— (0)	461 ± 44 (6)	610 ± 39 (6)

[1] Supplements begun on 19th day.

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

[3] Number surviving given within parentheses.

in parts by weight: niacin, 20; *p*-aminobenzoic acid, 10; calcium pantothenate, 8; riboflavin, 3; thiamine, 2; pyridoxine, 2; folic acid, 1; biotin, 0.1; vitamin B_{12}, 0.004; and sucrose (diluent), 200. Each animal received two drops per week of a fat-soluble vitamin concentrate containing haliver oil,[6] 20 parts (vol.); α-tocopherol, 7.2 parts (wt.); menadione, 0.120 part (wt.); and corn oil to make 50 parts (vol.). This represents 1020 I. U. of vitamin A, 10.2 I.U. of vitamin D, 7.2 mg of α-tocopherol and 0.12 mg of menadione per animal per week.

Sixty two- to 4-day-old guinea pigs[7] of both sexes were taught to eat the purified diet, and allowed to adapt to it over a 21-day period. During the adaptation period, each guinea pig received daily, by pipet, 1 mg of ascorbic acid per 100 gm body weight, administered as a 1% solution sweetened with sucrose.

The first 17 days of this period were used to test whether inositol above the level of 0.5 mg per 100 gm of diet[8] had any stimulating effect on the growth of guinea pigs receiving an otherwise adequate purified diet. To this end, the animals were divided into two groups, and *myo*-inositol was added to the diet of one group at a level of 200 mg per 100 gm. The other group served as a control.

The growth data obtained during this first period of the experiment are presented in table 2. It will be seen that the average weights of the two groups at no time differed significantly.

Beginning with the 18th day, all surviving animals were given the basal diet without inositol, and the vitamin C supplements were stopped on the 22nd day. For the final phase of the experiment, an effort was made to use animals which had lost 50 gm as a result of vitamin C depletion. The time required to achieve this depletion varied with individual animals from 13 to 18 days. The experimental groups (5 animals

[6] Abbott's Haliver Oil Simple.

[7] Obtained from Zeimet Bio-Farms, Madison, Wisconsin.

[8] The basal diet contained 0.5 mg of inositol per 100 gm as determined by microbiological assay.

each) were thus made up over a period of 6 days, as animals of approximately the desired degree of depletion became available. The regimens used, and the results obtained, are recorded in table 3. Supplements were again administered by pipet—inositol as a 10% solution and ascorbic acid as a sweetened solution of concentration suitable to the dose level involved.

As was expected (Collins and Elvehjem, '58) the survival of the guinea pigs receiving ascorbic acid supplements of

TABLE 2

Experiment 3

The effect of myo-inositol on the growth of guinea pigs fed an otherwise complete purified diet starting at two to 4 days of age

DAYS ON EXPERIMENT	AVERAGE WEIGHTS	
	Basal diet (23 animals) [1]	+200 mg % Inositol (26 animals) [1]
	gm	*gm*
1	105 ± 2 [2]	105 ± 3
5	100 ± 3	104 ± 4
9	112 ± 3	115 ± 4
13	127 ± 4	136 ± 5
17	147 ± 4	157 ± 5

[1] Only the weights of those animals surviving at the end of 17 days are included.
[2] Standard error.

0.1 mg per 100 gm body weight per day (groups II and III) was poor but better than that usually observed with no vitamin C. This level of the vitamin, however, was not sufficient to reverse the decline in the weights of the animals. A level of 0.2 mg of ascorbic acid per 100 gm body weight per day (groups IV and V) was also suboptimal in that some deaths from scurvy occurred and the time required for reversal of the weight decline in the survivors was longer than when larger amounts of ascorbic acid were given.

The guinea pigs receiving 0.3 mg of ascorbic acid per 100 gm per day (groups VI and VII) recovered quickly and

TABLE 3

Experiment 3

The effect of myo-*inositol on the recovery and growth of depleted young guinea pigs receiving various levels of ascorbic acid*

DAYS ON SUPPLEMENT	AVERAGE WEIGHTS						
	Group I 1 mg AA [1]	Group II 0.1 mg AA	Group III 0.1 mg AA + 100 mg IN [1]	Group IV 0.2 mg AA	Group V 0.2 mg AA + 100 mg IN	Group VI 0.3 mg AA	Group VII 0.3 mg AA + 100 mg IN
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1	143 ± 9 [2] (5) [3]	183 ± 10 (5)	182 ± 4 (5)	172 ± 16 (5)	172 ± 8 (5)	176 ± 10 (5)	175 ± 6 (5)
4	138 ± 8 (5)	163 ± 4 (5)	156 ± 4 (5)	160 ± 7 (4)	160 ± 6 (5)	179 ± 12 (5)	160 ± 5 (5)
8	153 ± 15 (5)	149 ± 4 (5)	141 ± 6 (2)	157 ± 4 (3)	169 ± 9 (4)	188 ± 12 (5)	174 ± 5 (5)
12	181 ± 16 (5)	142 ± 5 (4)	139 ± 3 (2)	172 ± 9 (3)	187 ± 12 (3)	210 ± 14 (5)	201 ± 12 (5)
16	197 ± 20 (5)	150 ± 12 (3)	143 ± 5 (2)	189 ± 13 (3)	179 ± 19 (3)	227 ± 19 (5)	216 ± 15 (5)
18	199 ± 22 (5)	146 ± 17 (3)	147 ± 2 (2)	199 ± 13 (3)	189 ± 17 (3)	227 ± 21 (5)	214 ± 16 (5)
20	205 ± 22 (5)	— (0)	— (0)	207 ± 18 (3)	205 ± 20 (3)	249 ± 24 (4) [4]	217 ± 14 (5)

[1] AA = Ascorbic acid; IN = Inositol. Dosages are per 100 gm body weight per day.

[2] Standard error.

[3] Number surviving given within parentheses.

[4] One animal died of respiratory trouble.

grew well, and none died of scurvy. A level of 0.3 mg may thus be considered optimal in the present experiment. It has been reported by Collins and Elvehjem ('58) that in experiments of longer duration a level of 0.3 mg of ascorbic acid per 100 gm body weight per day is not sufficient to maintain the guinea pig. This insufficiency does not manifest itself, however, in experiments of the duration reported here.

It will be seen from the table that the administration of inositol in addition to vitamin C did not significantly affect the mortality, rate of recovery, or rate of growth observed at any of the three levels used.

It must be noted that the control animals (group I) did not grow at an optimal rate. The average weight of this group at the start of the supplementation period was significantly lower than the average weights of the other groups, and this could easily be the reason for the poorer growth rate. However, no deaths occurred in the control group and significant weight gains were observed sooner after supplementation than in groups II through V.

SUMMARY AND CONCLUSIONS

The possibility that *myo*-inositol can substitute for or spare ascorbic acid in the diet of guinea pigs has been examined in three ways. First, inositol was given to adult guinea pigs subsisting on a vitamin C-deficient purified diet—to some from the beginning of the experiment, to others after scurvy had developed. Effects on the rate of onset of the disease, severity of the symptoms, and survival time were looked for.

Second, essentially the same plan was followed with a scorbutigenic natural diet.

And third, young guinea pigs were adapted to a purified diet, allowed to become scorbutic, then supplied with levels of ascorbic acid that ranged from suboptimal to sufficient. Some of the animals on each of these levels of ascorbic acid were also given inositol, and effects on the rate of recovery, survival, and rate of growth were looked for.

Inositol did not have any detectable antiscorbutic activity in any of these experiments.

At the start of the third experiment, while all of the guinea pigs were receiving the purified diet plus adequate vitamin C, the effect of adding inositol to the diet was tested. In keeping with the recent findings of other investigators (Reid, '54) no growth stimulation was observed.

ACKNOWLEDGMENTS

The authors wish to thank Miss Macie Collins for much helpful advice in planning the experiments, and Mr. John Martinson and Mrs. Carrie Gibson for technical assistance. The crystalline vitamins, except inositol, used in this work were furnished by Merck Sharp and Dohme Research Laboratories.

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EFFECT OF DIETARY PROTEIN AND FAT ON CHANGES OF SERUM CHOLESTEROL IN MATURE BIRDS [1,2]

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It is generally recognized that a high-fat diet will elevate serum cholesterol levels (Keys and Anderson, '55), and recently the nature as well as the amount of dietary fat has been deemed important to hypercholesteremia (Kinsell et al., '52; Shapiro and Freedman, '55). However, as animals on high-caloric diets eat less food than those on low-caloric diets, a high-fat diet will tend to reduce the actual protein intake.

Many suggestions have been made of ways to prevent increases in serum cholesterol (Peterson et al., '53; Shapiro and Freedman, '55; Clarkson et al., '56), although the greatest interest has been in means by which these values can be decreased most rapidly in hypercholesteremia.

Studies on experimental atherosclerosis in animals have shown that serum cholesterol can be increased by including fat and 1% or more of cholesterol in the diet (Katz and Stamler, '53). When cholesterol was no longer fed, an immediate decrease in serum cholesterol was noted. These results were

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[2] Portion of a thesis to be presented by M. Kokatnur as partial fulfillment of the requirement for the degree of Doctor of Philosophy in Food Technology. Presented in part at the meetings of the Federation of American Societies for Experimental Biology, Chicago, April 18, 1957 (Fed. Proc., *16:* 389. 1957).

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interpreted to indicate that the elimination of fat and cholesterol-containing foods from the diet would lower serum cholesterol levels.

In the present work the effect of dietary fat and protein on serum cholesterol levels was studied with mature male birds. Furthermore, it was of interest to note whether protein or fat was more significant as a contributing factor in decreasing serum cholesterol in hypercholesteremic chickens.

EXPERIMENTAL

Seventy-two Single Comb White Leghorn male birds 12 to 18 months of age were divided into two groups of 36 birds each and kept in feeder batteries for a 10-day pre-experimental period. One group was fed the basal diet given in table 1; the other received this diet supplemented with 1% of cholesterol. At the end of the 10-day period, 24 birds were selected from each group on a weight basis for the experimental period of 21 days. Five milliliters of blood were obtained from each selected bird by heart puncture. The birds

TABLE 1

Composition of the basal diet [1] used for all birds during the 10-day pre-experimental period

INGREDIENT	AMOUNT
	%
Cerelose	57.65
Soybean meal	30.00
Corn oil [2]	5.00
Bone meal	4.00
Distillers dried solubles	2.50
Vitamin A and D oil (3000A, 600D)	0.10
Iodized salt	0.50
Manganese sulfate	0.25
Vitamins [3]	+
	100

[1] With 1% cholesterol for 36 out of 72 birds for this 10-day pre-experimental period.

[2] Kindly supplied by Corn Products Refining Company, Argo, Illinois.

[3] Menadione, 0.4; riboflavin, 2.2; calcium pantothenate, 4.5; niacin, 25.0 mg/kg ration.

were then randomly distributed into 6 groups of 8 birds each (4 from the group fed the basal diet alone and 4 from the group fed the basal diet supplemented with 1% of cholesterol) and were kept for 21 days on modifications of the basal diet, as indicated in table 2. These diets were varied at the expense of the cerelose and the corn oil in the basal diet (table 1). Corn oil was chosen as the fat since it supplied an optimum amount of essential fatty acids and was also free of cholesterol. Comparisons were made between (a) diets containing 0.1% of fat and 7.5, 15 or 30% of protein and (b) those containing 15% of fat and the same levels of protein.

TABLE 2

Protein, fat and energy content of the 6 experimental diets

DIET	PROTEIN [1]	FAT [1]	ENERGY
	%	%	*Cal./gm*
1	7.5	0.1	3.7
2	15.0	0.1	3.4
3	30.0	0.1	3.0
4	7.5	15.0	4.4
5	15.0	15.0	4.1
6	30.0	15.0	3.7

[1] The protein (solvent extracted soybean meal, 50% protein) and the fat levels were varied at expense of cerelose and corn oil in the basal diet listed in table 1.

The gross energy values of the diets are given in table 2. The grams of protein consumed per bird were calculated for each group (table 3). Five milliliters of blood were obtained by heart puncture after the birds had been on the experimental diets for 10 days and also at the end of the 21st day of the experimental period. All of the blood samples were centrifuged individually and the serum stored at —10°C until ready for analysis. The total cholesterol values of individual samples were determined in duplicate by the Schoenheimer and Sperry method ('34) and the values obtained were subjected to a statistical analysis (Duncan, '55).

TABLE 3

Comparison of total feed and actual protein intake with changes in serum cholesterol values

DIETARY PROTEIN LEVEL	0.1% CORN OIL					15% CORN OIL				
	Total [1] feed intake	Actual [1] protein intake	Serum cholesterol			Total [1] feed intake	Actual [1] protein intake	Serum cholesterol		
			0 days	10 days	21 days			0 days	10 days	21 days
%	*kg*	*gm*	*mg %*	*mg %*	*mg %*	*kg*	*gm*	*mg %*	*mg %*	*mg %*
7.5	2.0	150	94	138	158	1.7	128	88	171	199
15.0	2.3	345	85	103	92	2.0	300	92	122	123
30.0	2.6	780	85	95	91	2.4	720	88	104	100

[1] Average intake per bird for 21-day experimental period. Least significant difference in serum cholesterol changes = 40 mg % at the 5% and 45 mg % at the 1% level.

TABLE 4

Comparison of protein intake with decrease in cholesterol values in serum from hypercholesteremic birds

PROTEIN LEVEL	0.1% CORN OIL				15% CORN OIL			
	Actual protein intake	Serum cholesterol			Actual protein intake	Serum cholesterol [1]		
		0 days	10 days	21 days		0 days	10 days	21 days
%	*gm*	*mg %*	*mg %*	*mg %*	*gm*	*mg %*	*mg %*	*mg %*
7.5	150	210	164	134	128	227	165	157
15.0	345	279	139	127	300	309	150	120
30.0	780	320	102	98	720	276	110	91

[1] Average starting cholesterol level = 273 mg %. Least significant difference in serum cholesterol changes = 70 mg % at the 5% level.

RESULTS

In agreement with the observations of other workers (Katz and Stamler, '53; Steiner and Dayton, '56) we noted that serum cholesterol values increased more rapidly in birds which had been fed fat than in those on an essentially fat-free diet (table 3). The serum cholesterol values of the birds which had been fed 15% of corn oil and 7.5% of protein increased 83 mg % in 10 days and another 28 mg % before the 21st day of the experimental period or from 88 to 171 and 199 mg % respectively.

A statistical analysis of these data indicated that the high-fat diet had increased serum cholesterol values significantly but only when the level of dietary protein was inadequate. At a level of 15% fat and 7.5% protein the serum cholesterol value had increased 111 mg % in 21 days but had not increased significantly in birds on a level of 15 or 30% protein. When the level of dietary protein was inadequate a significant increase of 64 mg % was also noted in the birds on the low-fat diet but this increase in serum cholesterol was significantly lower than the 111 mg % which was noted in those on the high-fat diet. A numerical but not a statistically significant increase in serum cholesterol was noted in the birds which had received 15% of corn oil as compared with those on 0.1% of corn oil at the 15 and 30% protein level.

An inverse linear relationship seemed to exist between the serum cholesterol level and the absolute intake of protein. The largest increase in serum cholesterol was noted in birds which had consumed the least amount of protein. No apparent relationship existed between serum cholesterol and differences in caloric (calculated metabolizable energy) intake or differences in the percentage of calories supplied by dietary fat when the protein intake was high or adequate.

It was noted that serum cholesterol values decreased most rapidly in hypercholesteremic birds when diets adequate or high in protein were fed (table 4). The serum cholesterol values of hypercholesteremic birds which had been fed 15% of protein decreased 140 mg % in 10 days as compared with

only 46 mg % for those fed 7.5% of protein. An increase in dietary protein improved these results still further. A drop of 218 mg % was noted in the cholesterol level of serum from hypercholesteremic birds fed 30% of protein, although this drop may have been exaggerated because of the higher average initial cholesterol level of this group. A statistical analysis of these data indicated that a significant drop in the cholesterol level of the serum from hypercholesteremic birds occurred in both the absence as well as the presence of dietary fat but that the decrease in serum cholesterol was more closely related to dietary protein than to dietary fat intake.

DISCUSSION

The present results indicated that a reduction in serum cholesterol may be produced more efficiently by increasing the dietary protein intake than by decreasing the intake of fat. It would seem, therefore, that a drop in serum cholesterol may occur by eliminating fat from the diet if foods high in protein are substituted for those high in fat. However, if this substitution is done blindly without regard for protein, the elimination of high-fat-containing food items may not effect serum cholesterol values significantly.

It has been reported (Gordon et al., '53) that an unidentified factor in sheep brain, freed of lipids and cholesterol, was effective in reducing serum cholesterol levels in cockerels. The level of dietary protein may have been a very important factor in these experiments, as brain residue is rich in protein and was added to a diet which was already adequate in protein. Later studies (Jones et al., '53), seemed to have shown this clearly. A cerebroside-lipid-cholesterol-free brain fraction showed a depressing effect on serum cholesterol which was believed to be due to the high level of protein in the diet.

SUMMARY

Male chickens 12 to 18 months of age were kept for 21 days on diets which contained 7.5, 15 or 30% of protein and 0.1 or 15% of corn oil. Samples of blood were obtained from each

bird by heart puncture at the beginning, on the 10th and on the 21st day of the experimental period and the serum cholesterol level of each sample was determined by the Schoenheimer-Sperry method. All data were subjected to a statistical analysis. The largest increase in serum cholesterol was noted in birds which had consumed the least amount of protein. No apparent relationship existed between serum cholesterol and differences in feed intake or differences in the percentage of calories supplied by dietary fat. The serum cholesterol levels of hypercholesteremic birds dropped rapidly during the three-week experimental period but it did not drop to normal values in this period of time unless the protein level was high.

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THE RIBOFLAVIN ECONOMY OF THE RAT

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In order to determine the optimum requirement of an essential nutrient under all circumstances, it seems necessary to understand first the biochemical economy of that nutrient — the interrelationships of intake, tissue concentration, rate of destruction and excretion, and the relationship between tissue concentrations and function.

It is the purpose of this paper to evaluate some of these relationships. Data are presented on: (1) the changes with time in the concentration of riboflavin in liver, and in the rest of the carcass of the rat with very low, moderate and high intakes of riboflavin, (2) the optimum tissue concentrations for maximum growth, (3) the expenditure of riboflavin necessary to maintain tissue at various riboflavin levels, (4) the fate of large doses of riboflavin and (5) the effect of varying the metabolic mixture or increasing total metabolism on riboflavin destruction.

MATERIALS AND METHODS

Nearly 400 white rats of the Wistar strain were used. Whenever possible litter mates and both sexes were divided equally among experimental groups, and litters of nearly equal size were employed.

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Diets and care of animals. Unless otherwise indicated the diets contained 18% of vitamin-free casein,[2] 8% of peanut oil, 20% of glucose, 49% of cornstarch, 3% of a modified Osborne and Mendel salt mixture (Hawk and Oser, '31) and 2% of cod liver oil. Each gram of diet was made to contain 5 μg thiamine, 6 μg calcium panthenate, 6 μg pyridoxine and 750 μg choline. Alternatively, in some cases, the vitamins were given as a supplement. In most instances the rats were fasted overnight before the beginning of an experiment to permit measurement of an initial weight that would not be unduly affected by variations in the contents of the gastrointestinal tract. The animals were individually caged over wide-mesh screen.

Preparation of the rats for analysis. Rats were placed on a riboflavin-free diet for 18 hours just prior to sacrifice. As much blood as possible was removed under anaesthesia from the exposed heart to minimize the otherwise large and variable blood content of the liver. Most of the hair was clipped off and the contents of the gastrointestinal tract were discarded to eliminate unabsorbed or bacterial riboflavin. The weight after this treatment was more reproducible than the live weight. Neither blood nor hair contain riboflavin in amounts significant compared to the total. Whole body concentrations of riboflavin have been calculated on the basis of this net carcass weight.

The liver was blended in a Waring Blendor with 9 volumes of 0.1 N HCl, then autoclaved 15 minutes at 15 pounds pressure. The rest of the carcass was ground in a meat grinder, transferred quantitatively to a Waring Blendor and blended with a mixture of crushed ice and distilled water. The blended mixture was diluted to 10 times the weight of the fresh tissue. This preparation was autoclaved 15 minutes at 15 pounds pressure after addition of 0.9 ml of concentrated HCl per 100 gm of solution (final concentration 0.1 N HCl). Aliquots of the autoclaved samples were neutralized with either 2 N sodium acetate or 2 N K_2HPO_4 (1 ml per 10 ml of

[2] Smaco.

blend), and centrifuged. Shaking with a little chloroform before centrifuging facilitated preparation of clear extracts in the case of fatty samples.

Riboflavin analysis. Riboflavin was measured in duplicate.[3] One- or two-milliliter aliquots of the liver extracts or 4 to 8 ml of the carcass extracts were diluted to 8 ml for analysis. Readings were made (1) after reduction with 0.1 ml of 5% sodium hydrosulfite in 5% $NaHCO_3$, (2) after reoxidation by bubbling oxygen through the tubes and (3) after addition of 0.05 to 0.15 ml of standard riboflavin solution (20 μg riboflavin per milliliter). Duplicate analyses concurred within 2 to 5%.

Microbiological *(L.casei)* assays made on a number of extracts gave results which agreed with fluorometric values within limits of reproducibility of the two methods. It was later found by Bessey et al. ('49) that riboflavin added to blended tissue was extracted only to the extent of 85 or 90%, and it required much higher dilution of tissue (1:50 or even 1:100) to remove all of the vitamin. Since recoveries of added riboflavin at tissue dilutions used (1:10) were quite uniform, and since the technique was kept constant throughout, it is felt that the data to be presented are valid except for being 10 or 15% too low.

The riboflavin content in most instances has been expressed on the basis of fresh weight. In a number of experiments, total nitrogen (in the liver) or alkali-soluble nitrogen (in the carcass) was also used as a basis for expressing the riboflavin content. However, since this did not alter the essential nature of the results, these figures have not been included except in the case of analyses of liver with widely differing riboflavin intakes (fig. 2).

RESULTS

Dietary intake of riboflavin and tissue concentration. With no riboflavin in the diet the vitamin concentration in the liver-free carcass fell rapidly at first, then more slowly, and

[3] Coleman photofluorometer, model 12.

finally leveled off after about three weeks at approximately half of the initial value (fig. 1). With 10 to 20 μg of riboflavin per day, the fall was slower but eventually the concentration fell to nearly the same low level. With 40 to 160 μg per day there was a moderate fall during the first three days with no further decrease.

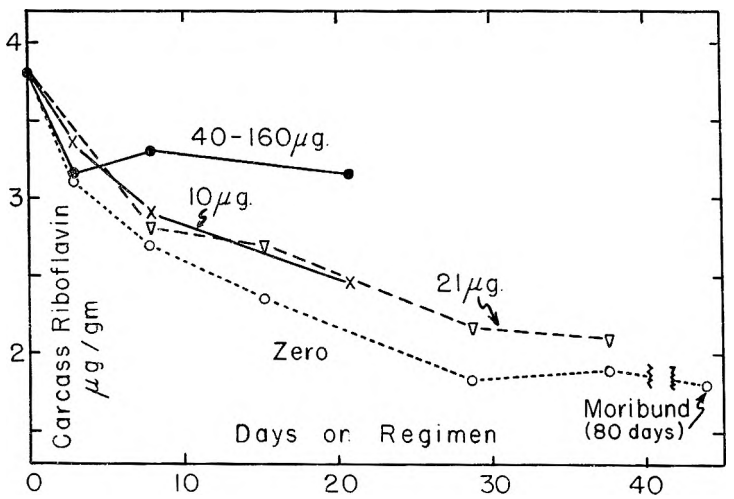

Fig. 1 Relationship between time on different levels of riboflavin intake and riboflavin concentration in the carcass (liver-free). The daily intakes of riboflavin (given as a supplement) are indicated (μg per day). Weanling rats of about 55 gm initial weight were used. The 40 to 160-μg group consisted of 27 animals in three groups each receiving 40, 80, or 160 μg of riboflavin per day. No significant differences in riboflavin concentrations were found among the three groups. Data from analyses of 87 rats are represented.

The riboflavin values in the *livers* of these rats have been calculated on the basis of protein (Kjeldahl N × 6.25) because of the marked increase in protein content of the liver which is known to occur in severe deficiency. Initially the livers contained 16.2% protein. After 21 days the average values were 21.0, 19.2, and 17.6% respectively with 0, 10 to 21, and 40 to 160 μg of riboflavin per day.

The riboflavin changes in liver (fig. 2) were similar to those in the rest of the carcass except that with a riboflavin-free

diet a somewhat greater percentage of fall occurred. Furthermore, with 10 and 21 μg of riboflavin per day the amounts of vitamin in the tissue tended to level off at intermediate concentrations.

Since variation in riboflavin intake affects growth as well as tissue concentrations, the total riboflavin per rat after three

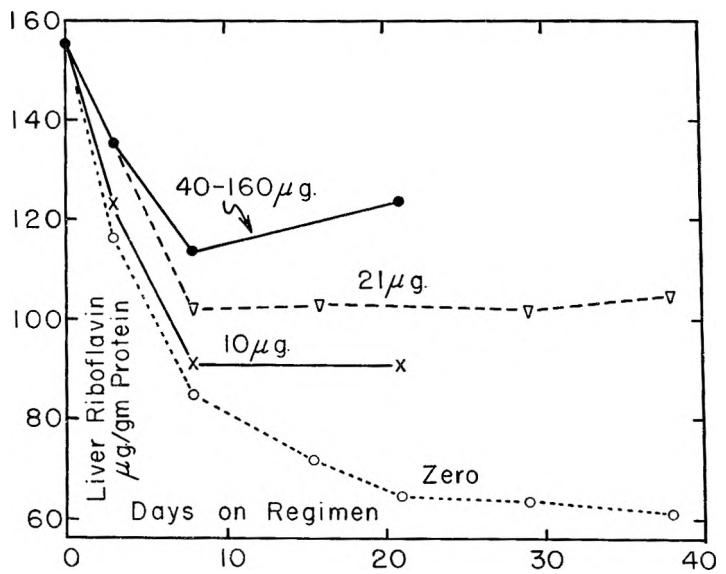

Fig. 2 Relationship between time on different levels of riboflavin intake and riboflavin concentration in the liver. These are the same rats as in figure 1. Based on wet weight rather than protein the values at 21 days were 12.1, 16.9, 20.0, and 22.2 μg per gram respectively with 0, 10, 21 and 40 to 160 μg of riboflavin per day, compared to an initial value of 25.5 μg per gram.

or 4 weeks on the experimental diets increased 60% with an intake of 40 to 160 μg per day, remained stationary with 10 to 20 μg per day, and fell to half with no dietary riboflavin.

The decrease in riboflavin of both liver and carcass with the largest intakes of the vitamin is partly due to the fact that the vitamin was given as a daily supplement. When riboflavin was incorporated into the diet less fall occurred. However, rats receiving a mixed diet [4] plus 160 μg of riboflavin daily

[4] Purina Chow.

retained more riboflavin in their tissues than those receiving the purified diet. It seems evident that there are factors other than riboflavin intake which can affect the maximum riboflavin content of tissues.

The shape of the time curves on a completely deficient diet suggests that part of the riboflavin is more readily excreted or destroyed than the remainder. This would be the case if part of the riboflavin were held in the tissues in dissociable combinations and if only the dissociated fractions were available for excretion or destruction. Warburg and Christian ('38) and others have reported well-defined dissociation constants for a number of flavoproteins. The fact that different flavoproteins vary in their dissociation constants and some do not seem to be dissociable at all, would explain the tendency for the tissue flavin concentration to approach an intermediate floor level rather than zero. After the tissue concentration reaches this floor value the rat loses weight sufficient to compensate for the inexorable daily destruction of riboflavin and thereby protects itself for a time from more severe tissue deficiency.

It is to be noted that for both liver and carcass there is not only a floor but also a ceiling above which it does not seem possible to push the riboflavin concentration. The ceiling possibly represents the level at which flavin enzymes have their full complement of flavin dinucleotide and mononucleotide.

The presence of rather well-defined maxima and minima for tissue riboflavin concentrations is one of the salient features of riboflavin nutrition as previous experimenters have pointed out (Kuhn et al., '35; Vivanco, '35; Fraser et al., '40; Van Duyne and Sherman, '41). The margin between maximum and minimum is narrow but the margin between maximum and suboptimum is still narrower, as will be shown.

Optimum riboflavin concentration of the tissues. An attempt was made to establish the optimum riboflavin tissue concentration. Growth was used as the basis of judgment, since it is one of the most sensitive criteria of nutritional

inadequacies. Therefore a diet optimum for growth might be expected to be optimum for other biological functions as well.

Ninety rats with an average weight of 54 gm were given riboflavin at 6 different levels. To avoid, if possible, the danger that something other than riboflavin might limit growth, the purified diet was supplemented with 25% by

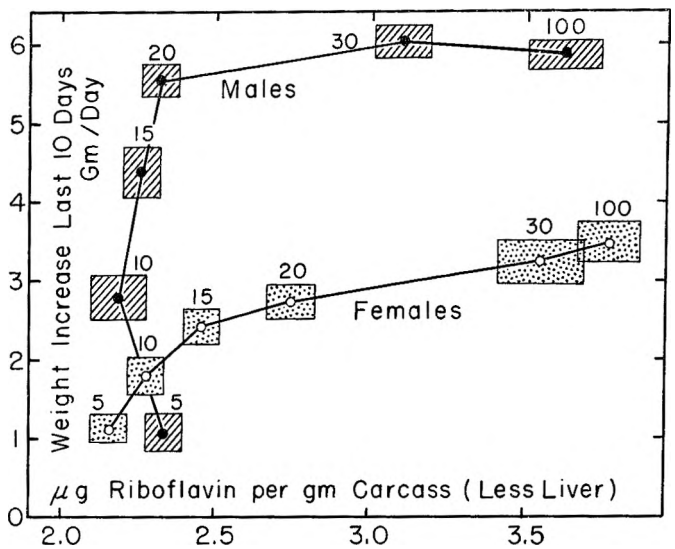

Fig. 3 Daily weight increase compared to the riboflavin concentration of the liver-free carcass. The boundaries of the rectangles are drawn at a distance from each point equal to one standard error of the mean. The numbers over the rectangles indicate the micrograms of riboflavin per 10 gm diet or approximately the daily intake.

weight of a commercial chow, which had been analyzed for riboflavin. Growth was excellent on this diet. With sufficient riboflavin, male rats grew an average of 6 gm per day.

As the dietary riboflavin was increased, the concentration in the male carcass did not change significantly until nearly maximum growth rate was attained (fig. 3). In marked contrast the female tissue concentration increased as growth increased. Presumably the greater stimulus to growth in the

male forced growth to the limit permitted by available riboflavin and by the lowest cell concentrations compatible with survival of the cells. It may be concluded that for maximum growth in these rats, riboflavin concentrations in the carcass had to be about 75% of the ceiling value in males, and 85% of the ceiling value in females.

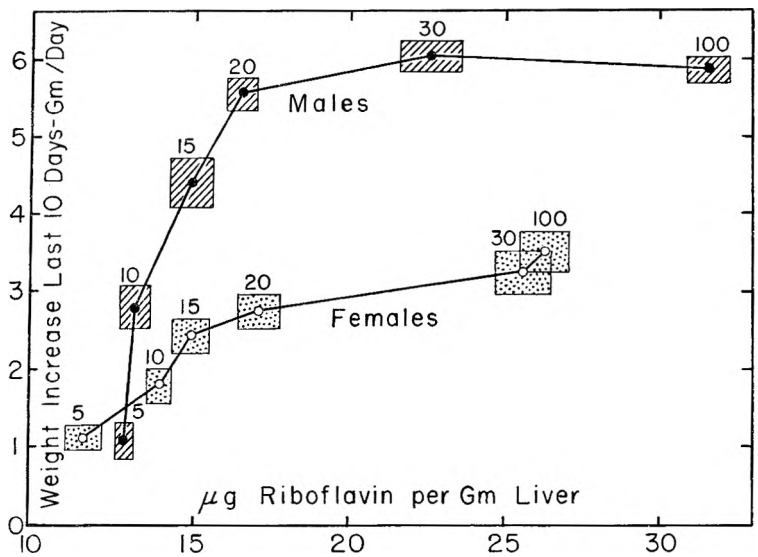

Fig. 4 Daily weight increase compared to the riboflavin concentration of the liver. The boundaries of the rectangles and the numbers over the rectangles have the same significance as in figure 3.

The concentration of riboflavin in liver (fig. 4) increased with both sexes as the growth rate increased in response to successively larger riboflavin intakes. Maximum growth appears to be associated with a liver concentration of not less than about 20μg of riboflavin per gram or approximately 65 and 75% of the maximum liver concentration for male and female respectively. The significantly higher maximum concentration in male liver is noteworthy and confirms the finding of Murray et al. ('46).

In general it would appear that growth is not demonstrably improved by tissue concentrations greater than about

75% of maximum. However, these data do not prove that average tissue levels below 75% of maximum in themselves constitute a handicap to growth since some single tissue, such as the pituitary, might actually be growth limiting. It is possible that the concentration in this single tissue would be the true governing factor.

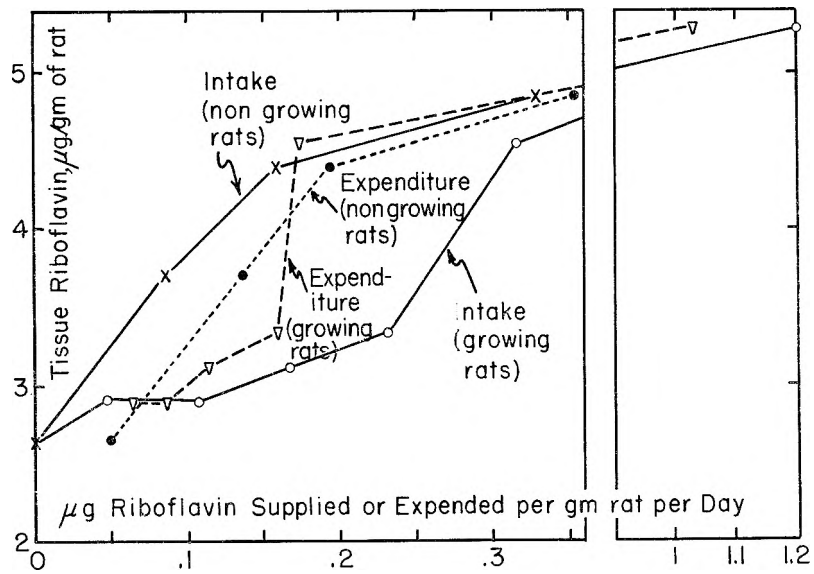

Fig. 5 Riboflavin intake, and expenditure versus riboflavin concentration in the rat. Expenditure of riboflavin is the daily intake plus the average riboflavin released per day from the tissues, or minus the average riboflavin taken up per day by the tissues, as the case might be.

The maintenance requirement. The vitamin supplied to a growing animal is used in part to furnish riboflavin for new tissue, and in part to maintain existing tissue. In order to separate the requirement into maintenance and growth components, a group of rats was given varying levels of riboflavin while receiving just sufficient food to sustain the initial weights. The results are compared (fig. 5) with the comparable values for the growing rats of figures 3 and 4. As anticipated, the non-growing rats required less riboflavin to

maintain a given tissue concentration than growing rats. Since the non-growing rats lost riboflavin, the total riboflavin expenditure is equal to dietary vitamin plus riboflavin released from the tissue (calculated from the difference between final and initial tissue concentrations). This curve of riboflavin expenditure (fig. 5) represents the maintenance requirement uncomplicated by growth.

The growing rats retained riboflavin except with the lowest intake of vitamin. The amount retained can be calculated from the weight increments and tissue concentrations and this deducted from the intake yields the expenditure of riboflavin (fig. 5). It will be seen that the amount of riboflavin used up by growing and nongrowing rats is roughly the same, which indicates that the maintenance component of the requirement is not materially affected by growth.

The data in figure 5 permit calculation of the riboflavin required to maintain tissues of a growing rat at any desired riboflavin concentration. Thus, in order to maintain the tissues of a 100 gm rat, which is growing 3 gm per day, at a level of 4 μg of riboflavin per gram, it would be necessary to furnish 100×0.16 for maintenance plus 3×4 for growth $= 28$ μg of riboflavin per day.

The nature of the riboflavin maintenance requirement. Urinary excretion accounts for very little of the maintenance requirement, since it was found that only negligible amounts of riboflavin appeared in the urine until very high levels of intake were reached. This agrees with the results of other investigators (Vivanco, '35; Van Duyne and Sherman, '41; Ferrebee and Weissman, '43; Sure and Ford, '43).

To demonstrate that large amounts of riboflavin may be destroyed in the body, approximately 500 μg of riboflavin were administered intraperitoneally to each of 6 young adult rats which had been maintained on a mixed diet plus 160 μg of riboflavin daily. After 24 hours on a riboflavin-free diet they were sacrificed. There was no increase in riboflavin content of either liver or carcass in spite of this large dose, and only 25% of the dose was recovered from urine, feces, and gastro-

intestinal contents. Therefore, 75% of the administered dose or 375 μg of riboflavin was destroyed (metabolized) during the 24 hours following its administration. Sure and Ford ('43) reported the destruction of at least this much roboflavin per day over a 30-day period when 1000 μg of riboflavin were given daily. This is about 20 times the destruction which would have occurred with more nearly physiological intakes, and about 100 times the minimum loss in severe riboflavin deficiency, indicated in our own experiments described below.

The question arises whether any significant part of the destruction of riboflavin is directly associated with its use. This may be investigated by measuring the loss of riboflavin to the body when metabolism is increased or when the metabolic mixture is varied to emphasize carbohydrate, fat, or protein metabolism. This latter might be expected to augment the riboflavin destruction if the vitamin were more rapidly destroyed during oxidation of a particular metabolite.

Evans et al. ('34) concluded from rat growth rates that fat does not have the same sparing action for riboflavin that it has for thiamine. Studies by Mannering et al. ('44) and Schweigert et al. ('45a) made it appear likely that the decrease in riboflavin requirement on a high dextrin diet (Mannering et al., '41) is ascribable to increased synthesis of riboflavin by intestinal flora.

Sherman and Derbigny ('32) reported that increasing the protein content of the diet from 6 to 12 to 18% resulted in progressively better weight gains on a riboflavin-poor diet. Since the proportion of fat and carbohydrate in the diet was only modestly reduced, it seems unlikely that this effect can be attributed to a riboflavin-sparing action from reduction in metabolism of either fat or carbohydrate. Sarett and Perlzweig ('43) found that with rats receiving about 10 μg of riboflavin per day an increase in dietary casein from 8 to 40% increased riboflavin concentration in the liver, but not in the rest of the carcass. Borgström and Hammersten ('44) have reported that increasing protein in the diet slightly *decreased* growth of rats on a low-riboflavin regime.

TABLE 1

Effect of variation in metabolic mixture and caloric consumption on loss of riboflavin

The rats received the experimental diets for 20 days, except for the last two groups which were 18 and 22 days respectively on diet.

	NO. OF RATS	CALORIES FROM			TOTAL INGESTED CALORIES	WEIGHT		RIBOFLAVIN			
		CHO	Fat	Protein		Initial	Change	Per gm	Per rat	Change [1] per rat	Change [1] per day
		%	%	%		gm	%	μg	μg	μg	μg
Series 1 [2]											
Initial	8					39.6		5.81	219		
Exp. A											
Low Fat	3	76	6	18	348	47.0	— 7	4.08	168	— 97	—4.8
Medium Fat	4	50	32	18	361	46.2	+13	3.74	185	— 75	—3.8
High Fat	4	23	59	18	362	46.8	— 1	3.96	174	— 90	—4.5
Exp. B											
Low Protein	6	80	17	3	368	42.0	—13	3.35	117	—117	—5.8
Medium Protein	6	74	17	9	359	42.5	+ 9	3.30	142	— 97	—4.8
High Protein	6	55	17	28	359	39.6	+20	3.39	147	— 78	—3.9
Exp. C											
Cold (12–15°C)	4	74	17	9	544	46.5	—16	4.24	156	—106	—5.3
Series 2 [2]											
Initial [3]	5					71.4		3.36	224		
High carbohydrate	5	81	9	10	490	71.3	+ 1 [4]	2.46	164	— 62	—3.1
High fat	5	32	61	7	(470) [5]	69.2	— 9 [4]	2.43	142	— 77	—3.8
High protein	5	42	9	49	(470) [5]	69.3	— 6 [4]	2.51	152	— 82	—4.1
Thyroxin [6]	4	81	9	10	616	63.8	—21 [4]	3.06 2.90	133	— 72	—3.6
Cold (12–15°C)	5	81	9	10	775	71.8	— 8 [4]	2.90	178	— 52	—2.6

[1] Calculated from initial weight and riboflavin of "initial" rats. Value is corrected for presence of 0.28 μg of riboflavin per gram of casein in the diet.

[2] Series 1 rats were transferred directly from stock diet to experimental diets; series 2 rats were kept two weeks on balanced riboflavin-free diet before being placed on experimental diets. In this series the experimental diets contained 2% of succinylsulfathiazole.

[3] After two weeks on the preliminary depletion diet.

[4] Calculated from weights after removal of gastrointestinal contents which were very bulky on the succinylsulfathiazole diets.

[5] Estimated.

[6] Thyroxin 0.1 to 0.2 mg given subcutaneously each day.

Because the records in the literature are inconclusive, the extent of riboflavin destruction was reinvestigated with (1) different metabolic mixtures and constant caloric intake, and (2) a constant metabolic mixture but with caloric consumption increased by thyroxine or cold environment (table 1).

To make it easier to detect specific changes in destruction rates, in a second series of experiments riboflavin was withheld for two weeks prior to the beginning of special treatment. In addition, succinylsulfathiazole was added to the diets to reduce possible riboflavin synthesis in the gastrointestinal tract. With an 18% casein diet (riboflavin-free) succinylsulfathiazole was found to decrease fecal excretion of riboflavin from 1.9 μg per day (three rats) to 0.5 μg per day (three rats). The sulfa drug likewise resulted in a significant decrease (12%) in average total riboflavin content of 5 rats maintained 26 days on a 30% casein diet (riboflavin-free) compared to 4 rats on the same diet without succinylsulfathiazole. The difference was equivalent to about 1 μg per 50-gm rat per day. Schweigert et al. (45a,b), also observed a decrease in riboflavin excretion when succinylsulfathiazole was added to the diet and found slightly less riboflavin in the liver when this drug was administered with a low-riboflavin diet.

In neither series (table 1) is there evidence of any marked effect on riboflavin destruction through changing the proportion of fat (6 to 61% of the calories) or protein (3 to 49% of the calories) or carbohydrate (23 to 81% of the calories). In the first series it might at first appear that protein had a sparing action on riboflavin, since the loss of riboflavin fell from 5.8 to 3.9μg per day as the protein calories were increased from 3 to 28% of the diet. However, the rats lost 13% in weight on the lowest protein level and gained 20% on the highest level. The faster growth of the high-protein rats would lower tissue concentrations early in the experiment to riboflavin levels at which losses would be diminished, as predicted from figures 1 and 2. The final riboflavin con-

centrations were about the same with each protein level.[5] In the second series the situation was reversed, apparently because the rats receiving 49% of their calories from protein lost weight compared to those receiving only 10% from protein. The relationship between change in weight and loss of riboflavin is apparent in all experiments with changing metabolic mixture and was in fact observed among individual rats on the same diet.

Increasing caloric expenditure produced by cold or by thyroxin administration failed to increase riboflavin loss. The data even suggest that considering weight losses, rats with higher caloric expenditure actually retain more riboflavin than expected. Thus rats of the second series kept in the cold and ingesting a total of 755 Cal. lost only two thirds as much riboflavin as the rats on high-fat or high-protein diets which received less than 500 Cal. These groups are comparable since they lost about the same amount of weight during the experiment.[6]

The above experiments indicate that if there is any true destruction of riboflavin "through use" it cannot be more than 25 μg per 1000 Cal. (0.01 μg per gram of rat per day). This is so small as to seem negligible in comparison with the probable requirement by man of about 500 μg of riboflavin per 1000 Cal. This is in striking contrast to the situation in regard to thiamine.

[5] Similarly, when an abundance of riboflavin was given (40 μg per gram of diet), tissue levels were found to be independent of the protein content of the diet. Thus with 3, 10, and 30% casein diets given for 20 days in approximately equal amounts, the average tissue levels were respectively 5.11, 5.24, and 500μg of riboflavin per gram of rat. These results agree with those of Schweigert et al. ('43) but disagree with Sarett and Perlzweig ('43) who found higher tissue concentrations with high protein diets. The latter workers gave only 33 μg of riboflavin per day, which may have been insufficient to produce ceiling tissue levels.

[6] Klein ('39a,b) found that feeding desiccated thyroid increased the activity of the flavoprotein D-amino acid oxidase in the liver. This suggests that in hyperthyroidism the liver, at least, may in fact be better prepared to retain riboflavin. Fontaine and Raffy ('42) found that rats maintained in a cold environment (2°C) had a 20% higher concentration of riboflavin in liver than control rats kept in a room at 27°C.

DISCUSSION

The data presented make it possible to give approximate values to the factors in the following equation for the rat:

(A) Riboflavin intake + (B) riboflavin released from tissues = (C) riboflavin deposited in tissues + (D) riboflavin excreted + (E) riboflavin destroyed through use + (F) riboflavin destroyed otherwise. The evidence presented indicates that little or no vitamin is directly destroyed as the result of its function in metabolism and that urinary excretion is almost negligible. Also, evidence from the literature indicates that fecal riboflavin is not of dietary origin. Hence items D and E of the above equation may be practically ignored in the rat. In man the urinary excretion is apparently of greater significance since, according to Melnick, Hochberg and Oser ('45), riboflavin of the order of 45% of the intake may appear in the urine. The chief loss of riboflavin to the rat is thus through destruction not specifically related to use. This destruction is exceedingly low for riboflavin-depleted rats — as little as 0.04 μg per gram of rat per day — and increases to about 0.2 μg per gram per day with tissue levels compatible with maximum growth. Above these levels destruction increases with intake. Thus an increasingly high price in riboflavin destruction (maintenance requirement) must be paid as levels are raised. Conversely, complete omission of dietary riboflavin is characterized by an initial rapid destruction of riboflavin which decreases markedly as tissue concentrations fall, thereby resulting in effective conservation of the vitamin.

This conservation is, of course, not effected without functional and eventually structural handicap. Optimum growth in the rat occurs with average tissue concentrations in the neighborhood of 75% of maximum, or about midway between the maximum level and the level reached in complete deficiency. The space between floor and ceiling is surprisingly small. For the whole rat the maximum is only twice the minimum.

The finding that destruction of riboflavin is not specifically associated with use leads to the interesting conclusion that

it is unnecessary to supply extra riboflavin when the caloric consumption is increased, as, for example, by physical exertion. It does not of course mean that a deficient animal would be as capable of doing hard work as a normal animal, nor does it indicate whether or not additional riboflavin would be required in other stress conditions, as, for example during pregnancy or in the presence of infection.

SUMMARY

1. The concentration of riboflavin in the liver and in the rest of the carcass has been measured for weanling rats maintained for three to 5 weeks with zero to 160 μg of riboflavin per day. With no dietary riboflavin tissue concentrations fall to a little less than half their initial value by the end of three weeks and then remain stationary. Maximum riboflavin concentrations are obtained with intakes of about 40 μg per day.

2. Maximum growth occurred with tissue riboflavin concentrations that were about 75% of the maximum levels attainable. Male rats grew more rapidly than females even with suboptimum tissue concentrations; hence with a given suboptimum intake of riboflavin, male tissues were lower in riboflavin than were female tissues.

3. It was found that when allowance was made for riboflavin deposited in the tissues or released from the tissues, as the case might be, that approximately the same expenditure of riboflavin was required by the growing rat as the non-growing rat to maintain a given tissue concentration of riboflavin. If, however, the gross intakes alone are compared, a rapidly growing rat may require up to three times as much riboflavin per gram as a non-growing rat to maintain a specific tissue level.

4. Destruction of riboflavin by the rat varies from as little as 0.04 μg per gram per day in severe deficiency to more than 6 μg per gram per day when a large excess of riboflavin is administered. Loss of riboflavin from the rat by excretion is negligible compared to destruction except with excessively

large levels of intake. In addition to any riboflavin deposited in the tissues, approximately 0.2 μg of riboflavin per gram per day is needed to balance destruction with tissue levels which permit maximum growth.

5. No increase in riboflavin destruction by riboflavin-deficient rats was observed when metabolism was increased by means of thyroxin or by a cold environment. Nor did changing the metabolic mixture to emphasize carbohydrate, protein or fat influence riboflavin destruction. Hence it is concluded that there is little or no direct destruction of riboflavin associated with its use.

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STEPWISE WEIGHT REDUCTION IN OBESE YOUNG MEN: NITROGEN, CALCIUM AND PHOSPHORUS BALANCES [1]

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Both clinical and metabolic studies of young women during weight reduction led to speculation as to the possible psychologic and physiologic advantages of the use of a scheme of stepwise weight reduction in which short periods of weight reduction would be alternated with periods of controlled weight maintenance until the desired objective was achieved (Young, '52a, b; Young et al., '53, '55). It has been observed clinically that a fair number of individuals can accept rigid caloric restriction over short periods of time but not for prolonged intervals. In some of these individuals if a "rest period" is allowed, restriction will again be accepted. There might, then, be a psychological advantage in planning for such respites in the reduction process. Also, metabolic studies with young obese women have shown that in the pre-reduction weight maintenance period all of the women retained calcium, phosphorus and nitrogen. After three to 4 weeks of weight reduction the subjects were either in equilibrium or still retaining the nutrients on the same level of intake; however, by the 8th to 10th week of the reduction period, the majority of the subjects were losing nutrients. Then by the third week

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TABLE 1

Age, height and weights of subjects during stepwise reduction

SUBJECT NO.	AGE	HEIGHT	INITIAL WEIGHT	IDEAL WEIGHT	EXCESS WEIGHT		SUBCUTANEOUS FAT [1]	NET WEIGHT LOST	EXCESS WEIGHT LOST	RATE OF WEIGHT LOSS
	yrs.	*in.*	*lbs.*	*lbs.*	*lbs.*	*%*	*mm*	*lbs.*	*%*	*lbs./wk.*
1	21	71.5	189.5	167	22.5	13.5	16	15.5	68.9	1.75
2	23	71.25	191.0	170	21.0	12.4	11	22.5	107.1	2.54
3	24	75.0	208.0	180	28.0	15.6	13	24.0	85.7	2.71
4	21	68.75	194.0	165	29.0	17.6	18	28.0	96.6	3.16
5	22	71.25	195.5	170	25.5	15.0	11	23.5	92.2	2.70
6	22	71.0	186.0	165	21.0	12.7	13	21.0	100.0	2.37
7	17	70.25	205.0	165	40.0	24.2	11	28.0	70.0	3.16
8	19	69.0	242.5	170	72.5	42.7	21	35.5	49.0	4.01
Range	17 to 24	68.75 to 75.0	186 to 242.5	165 to 180	21 to 72.5	12.4 to 42.7	11 to 21	15.5 to 35.5	49.0 to 107.1	1.75 to 4.01
Mean	21.1	71.0	201.4	169	32.4	19.2	14.3	24.8	83.7	2.80
Median	21.5	71.1	194.8	170	26.8	15.3	14.5	23.8	89.0	2.71

[1] Lateral aspect of thorax at 10th or 11th rib.

on a post-reduction weight maintenance diet there was an almost complete reversal of the downward trend and the subjects were in a state of equilibrium or retention with regard to all three nutrients (Young, '52b; Young et al., '53). The results of these two studies led us to wonder whether there might be physiologic advantages, as well, in the alternation of weight reduction and weight maintenance, since the losses of nutrients came only in prolongation of the reduction period. Hence, the present investigation was undertaken to study both the subjective reaction and the metabolic responses to weight reduction achieved by a stepwise process.

SUBJECTS AND METHODS

The 8 obese college men who served as subjects are described in table 1. Their excess weight ranged from 21 to 72.5 lbs. with a mean of 32.4. The percentage excess weight averaged 19.2 with a median of 15.3. Ideal weights were determined on the basis of body build using the Metropolitan Life Insurance Company Tables for Ideal Weights ('43). Body builds were estimated by a method developed by Showacre (Moore et al., '55). The only measure of body fatness available was that of the subcutaneous fat pads over the lateral aspect of the thorax at the level of the 10th or 11th rib as observed from chest x-rays. Subjects were carefully screened by interview and by psychological testing to select only those with sufficient emotional stability to cooperate in a 16-week controlled feeding period (Summerskill and Darling, '55).

The plan of the experiment included, in the following order: (1) an 18-day pre-reduction weight maintenance period; (2) a 24-day reduction period; (3) a 14-day maintenance period, a portion of which coincided with the spring semester vacation period; (4) a 22-day reduction phase; (5) a 13-day weight maintenance period; and finally (6) a 16-day reduction period (see table 2). Throughout the experiment, with the exception of the 10-day spring vacation, the men were weighed daily under uniform conditions.

Except for the period of spring vacation the men consumed a weighed diet prepared and served under the supervision of a dietitian at the Special Diet Table operated for research purposes. Only black tea, black coffee and water were allowed ad libitum and the quantities of these consumed were carefully recorded. Throughout the experiment the diet used was calculated to contain protein, 115 gm; calcium, 1.0 gm; phosphorus, 1.5 gm; and amounts of all other nutrients to meet the National Research Council's Recommended Dietary Allowances ('53). The caloric content of the diet was varied with the phase of the experiment (table 2) and caloric adjustments were made for weight maintenance of the individual men by the use of calories coming largely from sugar and butter combinations. Fifty percent of the 1800 Cal. in the reduction diet came from fat. During spring vacation the men were asked to control the quantities of milk, cheese, eggs and meat used.

Nitrogen, calcium and phosphorus balances were determined during 4 7-day periods. These occurred as follows: balance period I, the second week of pre-reduction weight maintenance; balance periods II and III, at the third week of the first and second reduction periods, respectively; and balance period IV, at the second week of the third reduction period.

During the balance periods all food intake was sampled as served. The 7-day composite was mixed, ground in a food chopper, weighed and frozen. The sample was then vacuum dried in a vacuum desiccator [2] and aliquots of the resulting powder were saved for analysis. Foods used for additional calories were similarly sampled and analyzed. Collections of urine were made for the 7 consecutive days of each balance period using toluene as a preservative. Stools for the balance period were marked by carmine and pooled. The nutrient contents of urine, feces and food aliquots were determined by the following methods: nitrogen, by the Kjeldahl method (Hawk et al., '47); calcium, by the micro-method of Kochak-

[2] Desivac.

TABLE 2

Caloric intakes and weight changes of subjects by period

SUBJECT NO.	PRE-REDUCTION MAINTENANCE (18 days)		REDUCTION (24 days)		MAINTENANCE (VACATION) (14 days)		REDUCTION (22 days)		MAINTENANCE (13 days)		REDUCTION (16 days)		ENTIRE PERIOD (107 days)
	Caloric intake	Weight change	Caloric intake	Weight change	Caloric intake [1]	Weight change	Caloric intake	Weight change	Caloric intake	Weight change	Caloric intake	Weight change	Weight change
	per day	*lbs.*	*per day*	*lbs.*	*per day*	*lbs.*	*per day*	*lbs.*	*per day*	*lbs.*	*per day*	*lbs.*	*lbs.*
1	3756	+0.5	1800	—12.0	—	+9.0	1800	—10.0	2963 [3]	+4.0 [3]	1800	—7.0	—15.5
2	3600	—0.5	1800	—13.5	—	+8.5	1800	—12.5	3223	+1.5	1800	—6.0	—22.5
3	3756	—1.5	1800	—14.5	—	+4.5	1800	— 9.0	3343	+3.5	1800	—7.0	—24.0
4	3600	—1.5	1800	—12.5	—	+3.0	1800	—10.0	3237	0	1800	—7.0	—28.0
5	3600	+0.75	1800	—12.25	—	+4.5	1800	—10.0	3131	0	1800	—6.5	—23.5
6	3600	+1.5	1800	—11.0	—	+4.0	1800	— 8.5	3182	+2.0	1800	—9.0	—21.0
7	3733	—2.5	1800	—15.0	—	+7.0	1800	—11.0	2928	+0.5	1800	—7.0	—28.0
8	3600 [2]	—5.75 [2]	1800	—12.25	—	+4.0	1800	—12.5	3197	0	1800	—9.0	—35.5
Range	3600 to 3756	+1.5 to —5.75		—11.0 to —15.0	—	+3.0 to +9.0		— 8.5 to —12.5	2928 to 3343 [3]	0 to 3.5		—6.0 to —9.0	—15.5 to —35.5
Mean	3664	—0.6	1800	—12.9	—	+5.6	1800	—10.4	3177 [3]	+1.1 [3]	1800	—7.3	—24.8
Median	3600	—0.5	1800	—12.4	—	+4.5	1800	—10.0	3199 [3]	+0.5 [3]	1800	—7.0	—23.75

[1] All subjects on vacation for 10 days—exact caloric intake unknown.

[2] Started experiment late; loss represents 4 days at intake given. Not included in averages.

[3] Calories uncontrolled on 4 day emergency leave. Subject not included in averages.

ian and Fox ('44); and phosphorus, by the colorimetric method of Koenig and Johnson ('42).

At the conclusion of the experiment the men were given a questionnaire in order to learn (a) their reaction to various phases of the experiment and (b) their food intake patterns.

RESULTS AND DISCUSSION

Weight loss and caloric intake

For the entire experiment net weight losses ranged from 15.5 to 35.5 lbs., with a mean of 24.8 (table 1). Forty-nine to 107% of the excess weight was lost; the median loss being 89%. The rate of weight loss ranged from 1.75 to 4.01 lbs./week with a mean of 2.8. Table 2 gives the weight changes of the subjects during the various periods of the experiment. It is obvious that in the approximately 9 weeks of caloric restriction the subjects actually lost considerably more weight than the net losses indicate. Total pounds lost ranged from 26.5 to 39.5; the median, 30.0; the mean, 31.3. Weight gains during spring vacation, and a few gains during the last maintenance period, caused net losses to be smaller than total losses.

During the 18-day pre-reduction weight "maintenance" period, several of the men were losing slightly on an intake of 3600 Cal. daily and required an increase in calories to prevent further losses. Three of the men gained slightly on the 3600 Cal. intake (table 2).

In the 14-day maintenance period which included 10 days of spring vacation the men showed apparent gains of three to 9 lbs., median, 4.5. Though some of the gains might reflect lack of cooperation, actually on a weighed intake of 3600 Cal. most men had showed increases of 3.5 to 4 lbs. during the 4-day period before vacation began. With the use of the high-protein reduction diet, in which 50% of the calories come from fat sources, it has been a common observation that at the beginning of each reduction period weight is lost at an accelerated rate. Then, with a shift to a "maintenance" diet

of greater total food, higher calories, and usually more carbohydrate, there is a sudden increase in weight which is promptly lost on return to the higher fat reduction diets. The bases of the weight fluctuations are not known, but they are greater than can be accounted for by either the changes in calories or in the weight of food consumed. The rapidity with which the weight variations occur would suggest hydration as the causative factor.

During the last weight maintenance period caloric intake was controlled. In some cases it was necessary to reduce intake to below 3000 Cal. in order to maintain weight. For 7 of the subjects on an average intake of 3177 Cal., the mean weight change was +1.1 lbs., the median +0.5. As has been reported previously for both men and women (Brown and Ohlson, '46; Young et al., '53, '57) weight reduction does definitely decrease caloric needs, due not only to a fall in the expenditure of basal metabolic energy and in the energy cost of activity, but perhaps to other unknown factors as well.

The total weight losses during the reduction periods were related to the intial weight of the subjects, those with the greatest initial weights losing the largest amounts almost in order of rank (subjects 8, 7, 3, 4 and 5). Three of these 4 men (3, 4 and 7) were also those with the greatest amount of physical activity. Subject 8, who was not active but who was by far the heaviest, clearly lost the most weight. Another point of interest is that, in this experiment, as in previous ones in which the high-protein, moderate-fat type of diet was used, there was a notable lack of "plateauing" in the weight reduction curves. (Young, '52a, b; Young et al., '53, '57).

The weight gains during the unsupervised vacation period, even when the men were sincerely trying to cooperate in maintaining their weights, are evidence that in any stepwise reduction scheme supervision and counseling are as essential during the weight maintenance periods as during the actual reduction phases.

TABLE 3

Mean daily retentions of nitrogen, calcium and phosphorus — by periods

BALANCE PERIOD	RETENTION BY SUBJECTS								MEAN INTAKE	SUMMARY		
	1	2	3	4	5	6	7	8		Number of subjects		
										R [1]	B [2]	L [3]
	gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*			
Nitrogen												
I. Pre-reduction, second week	+0.760B [2]	+1.486R [1]	+3.101R	+1.187R	+2.446R	+2.477R	+0.476B	—	19.728	5	2	—
II. Reduction, third week	—0.759B	—2.543L [3]	—3.514L	—1.597L	—1.492L	+0.871B	—3.984L	+ 6.031R	17.984	1	2	5
III. Reduction, 6th week	+0.949R	—3.292L	—3.458L	—1.098L	+0.676B	+0.844B	—4.314L	+10.524R	18.800	2	2	4
IV. Reduction, 8th week	—1.905L	—3.356L	—4.571L	—1.280L	—0.008B	—1.321L	—3.757L	+ 9.732R	19.286	1	1	6
Calcium												
I. Pre-reduction, second week	+0.153R	+0.338R	+0.394R	+0.074B	+0.221R	+0.298R	+0.162R	—	1.184	6	1	—
II. Reduction, third week	+0.138R	+0.195R	+0.182R	+0.006B	+0.027B	+0.315R	—0.164L	+ 0.418R	0.932	5	2	1
III. Reduction, 6th week	+0.003B	—0.131L	+0.269R	+0.083B	+0.117R	+0.092B	+0.102R	+ 0.486R	0.970	4	3	1
IV. Reduction, 8th week	—0.036B	+0.080B	+0.661R	—0.084B	—0.080B	+0.213R	—0.156L	+ 0.500R	1.043	3	4	1
Phosphorus												
I. Pre-reduction, second week	—0.029B	+0.137B	+0.158R	—0.044B	+0.105B	+0.088B	—0.004B	—	1.545	1	6	—
II. Reduction, third week	+0.100B	+0.009B	—0.082B	—0.095B	—0.026B	+0.203R	—0.158L	+ 0.529R	1.505	2	5	1
III. Reduction, 6th week	—0.179L	—0.486L	—0.256L	—0.141L	—0.005B	—0.035B	—0.124B	+ 0.682R	1.403	1	3	4
IV. Reduction, 8th week	—0.048B	—0.207L	+0.184R	—0.220L	+0.011B	+0.000B	—0.309L	+ 0.856R	1.573	1	3	3

[1] R = Retention greater than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

[2] B = Balance: retention or loss less than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

[3] L = Loss greater than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

Metabolic studies

In table 3 are presented the data for nitrogen, calcium and phosphorus for the 4 balance studies. For all three nutrients, in the pre-reduction weight maintenance period, all men were either in balance or retaining the nutrients. During the reduction periods the balance shifted from retention or balance toward balance or loss of the nutrients. This tendency was most marked for nitrogen and phosphorus, though it was also true to a lesser extent for calcium. These are the same tendencies noted in previous studies with men (Young et al., '57).

Nitrogen. Four subjects (2, 3, 4, 7), who retained nitrogen in the pre-reduction maintenance period, were consistently in negative nitrogen balance in all observations made during weight reduction. Three subjects (1, 5, 6) were either in a state of balance or retention with regard to nitrogen in all but one of the reduction periods. One subject (8) retained large quantities of nitrogen throughout the reduction studies. Unfortunately, since he joined the experiment late we do not have data for him during the maintenance period. He was much the heaviest man, lost the greatest amount of weight, and yet retained the greatest quantities of all the nutrients. No errors in technique or cooperation could be found. Strang et al. ('31) and Keeton and Dickson ('33) have reported apparent storage of nitrogen with consistent loss in weight in severely overweight individuals.

In every individual, in all of the reduction periods, there was a sharp increase in urinary nitrogen expressed as a percentage of intake over that found in the pre-reduction period. Especially was this true in the men who showed consistent negative balances. There was a definite decrease in fecal nitrogen, expressed as a percentage of intake in all reduction periods.

Nitrogen retention patterns were similar to those observed previously during straight reduction (Young et al., '57). It seems apparent that nothing was gained as far as maintaining nitrogen balance is concerned by the use of the stepwise reduction regimen.

The nitrogen balance performances of individual subjects were examined in relation to various factors. No relationship seemed to exist between the initial percentage of excess weight or the percentage of excess weight lost and the nitrogen balance. Subject 6, who lost 100% of his excess weight, was in nitrogen balance until the last period, while subject 7, who lost only 70% of his excess weight, was in negative nitrogen balance in all of the reduction balance periods. Obviously, a more precise indication of body composition and a true measure of body fatness is desirable for comparison with metabolic response. Nitrogen balance did appear to be related to the *total* pounds lost during the experiment, not the *net* pounds. In general those who lost the greatest number of pounds were the ones in negative nitrogen balance in all reduction periods (7, 2, 3, 4). The notable exception is no. 8, who lost a total of 40 lbs. and yet retained nitrogen throughout. Whether or not a subject had used more or less protein food before the experiment seemed to have little influence on his pattern of retention in any of the periods. Results of balances at the end of two weeks in the final reduction period did not seem to differ much from those at the end of three weeks in the other periods. Nor did the uncontrolled intake of the "maintenance" diet during spring vacation between periods II and III seem to give results in period III which differed essentially from those in periods II and IV after controlled maintenance.

Calcium. During the pre-reduction period 6 of the 7 men were retaining calcium; one was in calcium balance. In each successive reduction balance period, an increasing number of men went from calcium retention to balance, and in each period one individual was in negative balance. Subject 7 was in negative balance in periods II and IV; subject 2, in period III. The negative balances were associated with unusually high fecal excretions of calcium.

For the group as a whole there appeared to be a relationship between the retention of calcium in the pre-reduction period and the estimated previous daily milk intake. Subject 3, with previous intake of 16 oz. of milk per day retained 31.8%; sub-

ject 4, with 64 oz. retained only 6.1% of calcium. The retentions of subjects with intakes between these levels were, in general, proportional to the previous milk intake. Subject 8 was exceptional; he consistently retained large quantities of calcium in all periods.

The urinary excretion of calcium expressed as a percentage of intake increased in the reduction periods over what it was in the maintenance periods. For most subjects the percentage for each reduction period remained fairly uniform. Mean fecal excretion of calcium increased in the reduction periods over the pre-reduction value; there was no consistent pattern for individuals from period to period.

Phosphorus. Although during the reduction periods the overall patterns of phosphorus retentions showed the decline from maintenance that nitrogen did, the changes were neither as consistent nor as pronounced (table 3). Three subjects (5, 6, 8) were in a state of either phosphorus balance or retention throughout the experiment. Subject 8 retained large quantities of phosphorus in every period. For the other 5 men, phosphorus was being lost during one or more periods. In contrast to the response with respect to nitrogen, no subject consistently lost phosphorus during each reduction balance period. However, in general, the subjects who lost phosphorus were those who lost nitrogen, though not necessarily during the same balance period.

The phosphorus patterns were very similar to those shown by the men studied previously (Young et al., '57) except that there was less consistency in the patterns of the individuals. Hence, with respect to phosphorus there is no evidence of physiological advantage in the stepwise regimen.

Urinary excretion of phosphorus expressed as a percentage of intake tended to increase during the reduction periods, but the magnitude of increase was much smaller than that of nitrogen. Fecal excretion tended to increase in contrast to that of nitrogen.

Response to questionnaire

We were interested in the subjective reaction to the stepwise reduction regimen. Six of the 8 men felt they would prefer the stepwise procedure to uninterrupted weight reduction. Also, the supervising dietitians, who were in daily contact with the men, felt the relief offered by periodic easing of caloric restriction was very evident in the reactions of the men and was a large factor in the excellent cooperation obtained. One man was definitely against a stepwise plan because he found the adjustment to each new reduction period difficult. The 8th subject said frankly that 15 weeks of any rigidly controlled feeding regimen was too much, whether it involved caloric restriction or not. He felt uninterrupted reduction would be best since he could not accept controlled feeding without the reward of weight loss.

For the majority of subjects, then, the subjective reaction to a stepwise reduction scheme was good. However, it should be recognized that there can be some individuals who find continuous restriction easier. The two such subjects in this experiment were the ones who showed the greatest emotional reaction to restriction. They were more irritable and short-tempered, and there was grumbling during the periods of restriction.

SUMMARY

The effect of stepwise weight reduction on the nitrogen, calcium and phosphorus metabolism of 8 obese young men was studied by means of the balance technic. Seven-day balances were performed during pre-reduction weight maintenance and during the last week of each reduction period.

The net weight losses ranged from 15.5 to 35.5 lbs.; the mean being 24.8; the median, 23.8 lbs.

During the pre-reduction maintenance period all subjects were in equilibrium or retaining all three nutrients. With caloric restriction both nitrogen and phosphorus retention was decreased in most of the subjects. Fewer subjects showed poorer calcium retentions. Thus there was no evidence that

for men a stepwise reducing regimen eliminates the physiological shortcomings of an uninterrupted reduction regimen.

The most obese of the subjects, who also achieved the greatest weight losses, showed unique metabolic responses during all of the reduction balance periods. Consistently he was retaining large quantities of nitrogen, calcium and phosphorus.

From a psychological standpoint, 6 of the 8 men felt they would prefer stepwise reduction to an uninterrupted regimen. The two men who found caloric restriction most difficult felt they would have preferred uninterrupted weight reduction.

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INCORPORATION OF ACETATE-2-C^{14} INTO LIVER AND CARCASS LIPIDS AND CHOLESTEROL IN BIOTIN-DEFICIENT RATS [1,2,3]

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The mechanisms by which biotin functions in the intermediary metabolism of higher animals are not fully understood. Evidence that it is necessary for carboxylation and decarboxylation reactions has recently been reviewed by Lardy and Peanasky ('53). While oleic acid can replace biotin in the metabolism of certain lactic acid bacteria (Williams and Fieger, '46), it has not been shown to have biotin activity in the rat (Luckey et al., '55). The earlier view that biotin feeding might result in fatty livers has not proved valid (Best et al., '46). There is considerable evidence, however, that incipient or borderline biotin deficiency in the cholesterol-fed rat results in decreased accumulation of fat and cholesterol, even when a normal rate of food intake is maintained (Okey et al., '51).

Curran ('50) reported decreased synthesis of fat on the basis of studies with deuterium oxide fed to biotin-deficient rats. However, Guggenheim and Olson ('52), who fed ace-

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tate-1-C^{14}, found no differences in the incorporation of C^{14} into the fatty acids of pair-fed and biotin-deficient rats.

The present paper reports a study of the metabolism of injected acetate-2-C^{14} in biotin-deficient, pair-weighed control and ad libitum-fed control rats. Measurements were made of oxidation of C^{14} from the acetate and incorporation of C^{14} into liver lipid, free and total cholesterol, and glycogen. The incorporation of C^{14} into carcass lipid was also measured.

EXPERIMENTAL

Diets. Synthetic diets were fed: 5% vitamin-free casein, 15% egg albumin, 10% coconut oil, 66% sucrose, and 4% salts (USP XIV). Vitamin supplements, given three times weekly, supplied per day: 50 μg thiamine, 40 μg riboflavin, 20 μg pyridoxine, 100 μg calcium pantothenate, 66 μg niacin, 100 μg *p*-aminobenzoic acid, 20 μg folic acid, 49 μg menadione, 25 mg inositol, 10 mg choline, 100 I. U. vitamin A,[5] 10 I.U. irradiated ergosterol, and 0.5 mg α-tocopherol. In addition, control rats received 15 μg biotin per day.

Treatment of animals. Male rats of the Long-Evans strain were caged separately at weaning. One littermate received the biotin-deficient diet, a second, the control diet ad libitum, and a third was fed the control diet once daily in just sufficient amounts to support a weight gain equal to that of his deficient littermate. The first symptoms of biotin deficiency appeared in about three weeks, but amounted only to a poorly defined "unthriftiness." Loss of hair, decreased rate of gain, and emaciated appearance (lack of subcutaneous fat) developed gradually. Two preliminary series, consisting of 5 and of three rats, respectively, were sacrificed at 6 weeks. Data presented in this paper are for a larger series sacrificed during the 7th week on diet (series I, 18 rats) and for another series sacrificed during the 10th week on diet (series II, 23 rats). Deficiency symptoms were not much more severe in the latter than in the former series. Experience with the pre-

[5] Distillate, Eastman.

liminary series had shown that the pair-weighed animals ate their 24-hour food supply within two to 4 hours after feeding, and might therefore be considered to have fasted for 20 hours previous to autopsy. Since fasting decreases the rate of liver cholesterol synthesis (Lyon, Masri and Chaikoff, '52), and it was desirable to secure control as well as experimental rats which had eaten just prior to injection, all of the rats for which data are given were fasted for 12 hours and then allowed access to food for two hours before injection of the labeled acetate. They also had access to food in the interval between injection and autopsy.

Each rat was injected intraperitoneally with the indicated dose of labeled acetate, and placed in a glass metabolism cage for 4 hours. During this time, carbon dioxide-free air was drawn through the cage, and the exhaled carbon dioxide was collected in 2N sodium hydroxide. Carbon dioxide collections were made one half, one, two, three, and 4 hours after injection for series I. For series II, only the total excretion was determined. Four hours after injection the animals were sacrificed; the liver and carcass were frozen for future analyses.

Chemical analysis. Methods for chemical analysis of the liver lipids were those described by Okey and Lyman ('54). Glycogen was determined by the method of Seifter et al. ('50). Carcasses were air-dried at 50°C for 48 hours, extracted with alcohol and then with ether. The ether was evaporated by heating at 50°C, and the lipid was extracted from the alcohol-water residue with petroleum ether. Total lipid was determined gravimetrically.

Isotope analysis. Carbon dioxide-C^{14} in expired air was determined after precipitation of the carbon dioxide as barium carbonate (Brice and Okey, '56).

Liver lipid C^{14} was determined in an aliquot of the final petroleum ether extract of total liver lipid used for chemical analysis. The extract was measured directly into aluminum cups, the solvent evaporated, and the samples counted.

Free cholesterol C^{14} in the liver was determined in duplicate using 40- to 60-ml aliquots of the original alcohol-ether extract of the homogenized liver. These were evaporated to dryness, the lipid was dissolved in acetone-ether (1:2), and the cholesterol was as precipitated with an excess of digitonin. The precipitate was centrifuged, and the supernatant layer was discarded. After being washed three times with hot water, the digitonide was washed successively with acetone-absolute alcohol (1:1), with acetone-ethyl ether (1:2), and finally with ethyl ether. The digitonide was recrystallized from methanol, washed once with acetone, transferred to aluminum planchets from an acetone slurry, and counted. The counts per minute per milligram of cholesterol were calculated. The weight of the cholesterol was taken as 24.4% of that of the digitonide (theoretical).

Total cholesterol C^{14} from the liver was precipitated as digitonide after saponification and extraction with petroleum ether as for the chemical analysis. It was then subjected to the purification described above. Preliminary studies with non-radioactive cholesterol contaminated with palmitic acid-C^{14} had shown that this sequence of washing and reprecipitating would remove any radioactivity due to contamination with fatty acid.

Glycogen was precipitated, with alcohol, from duplicate aliquots of the potassium hydroxide hydrolyzate of liver. The precipitate was centrifuged, and dissolved in trichloracetic acid to remove protein. After centrifugation, the glycogen was reprecipitated twice with 95% ethanol. It was finally plated (from an acetone slurry) on weighed aluminum planchets and counted.

Carcass lipid C^{14} activity was determined directly after evaporation, on aluminum planchets, of appropriate aliquots of the petroleum ether extracts used for the chemical determination. Self-absorption corrections were made in the determination of the activities of barium carbonate, cholesterol digitonide, glycogen, and of carcass lipids.

A thin-window tube,[6] with helium bubbled through absolute ethanol at 0°C as the gas, was used for all counting.

RESULTS AND DISCUSSION

Typical growth curves for littermates injected during the 10th week on diet (series II) are shown in figure 1. Animals in series I showed similar growth. Mean gains per gram of

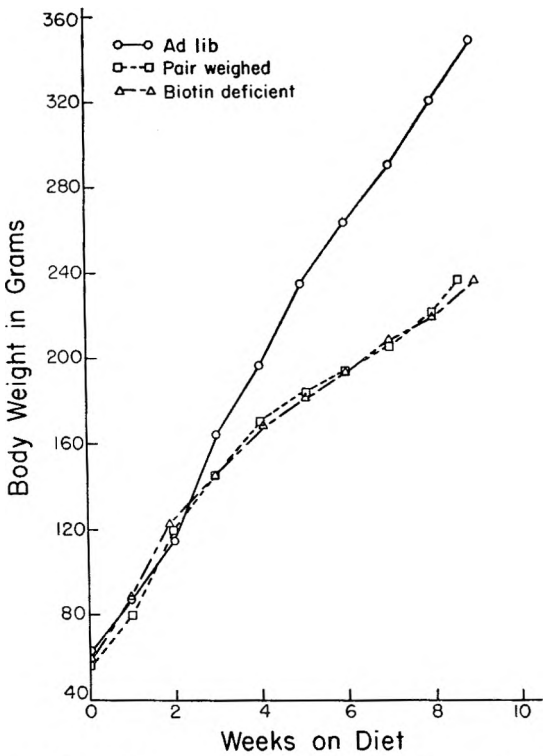

Fig. 1 Mean growth curves, rats of series II.

food eaten were 0.36, 0.28, and 0.23 gm at 7 weeks and 0.33, 0.29, and 0.26 gm at 10 weeks, for the ad libitum-fed, pair-weighed, and biotin-deficient animals, respectively. These figures would not, in themselves, indicate any greatly impaired food efficiency in the deficient rats.

[6] Mylar, du Pont.

Figure 2 shows cumulative excretion curves for $C^{14}O_2$, together with specific activity data for $C^{14}O_2$, for the rats of series I. The two preliminary series studied after 6 weeks on diet showed similar variations. For the 10-week-old animals of series II, percentages expired were 71.3 ± 1.3 [6] for the ad libitum-fed rats, 61.7 ± 2.8 for pair-weighed controls, and 68.7 ± 4.0 for the biotin-deficient rats. This meant that a significantly higher percentage of the injected activity was expired by the deficient rats studied at 7 weeks ($P < 0.001$),

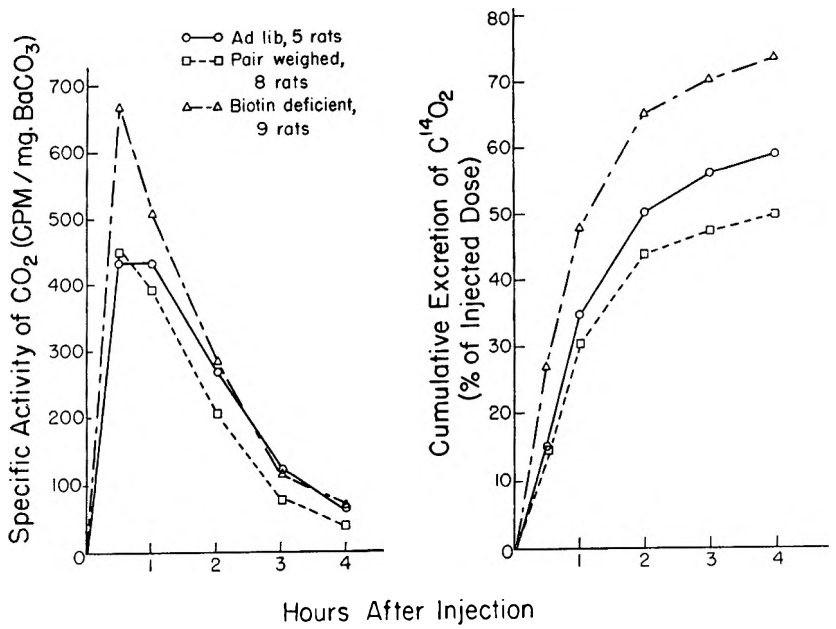

Fig. 2 Specific activity and cumulative excretion of $C^{14}O_2$ in typical littermate rats of series I.

but that at 10 weeks the differences were not significant. The fact that the 7-week controls were studied at the height of their adolescent increase in growth rate may have accounted for some of their extra retention of C^{14}. The specific activity of CO_2 from deficient animals was increased during the first hour. Hour-to-hour determinations of $C^{14}O_2$ activity were not made for the rats of series II.

Liver lipid and cholesterol data are shown in table 1, together with figures for carcass fat. Activity data for liver and carcass lipids are found in table 2. Total lipid concentrations tended to be only slightly higher, in the smaller livers of the normal but underfed rats of the pair-weighed groups, than in the ad libitum-fed deficient groups. Absolute amounts were highest in the larger livers of the ad libitum-fed controls.

TABLE 1

Liver [1] and carcass [2] lipids and liver cholesterol

	SERIES I (7-wk.)		SERIES II (10-wk.)	
Liver lipids:	*% moist wt.*	*mg*	*% moist wt.*	*mg*
Ad libitum	4.75 ± 1.06 [3]	415	3.43 ± 0.08	393
Pair-weighed	5.31 ± 0.73	333	4.39 ± 0.40	354
Deficient	4.46 ± 0.47	250	3.95 ± 0.27	349
Total cholesterol:				
Ad libitum	0.23 ± 0.011	20.4	0.22 ± 0.049	25.2
Pair-weighed	0.20 ± 0.009	13.0	0.24 ± 0.009	19.5
Deficient	0.22 ± 0.011	11.9	0.23 ± 0.014	20.3
Free cholesterol:				
Ad libitum	0.19 ± 0.006	16.4	0.18 ± 0.008	20.6
Pair-weighed	0.16 ± 0.006	10.3	0.18 ± 0.004	14.2
Deficient	0.18 ± 0.010	10.1	0.19 ± 0.007	16.7
Carcass lipid:				*gm*
Ad libitum			14.9	50.3
Pair-weighed			9.6	22.2
Deficient			9.1	20.4

[1] Number of animals: series I, ad libitum, 4; pair-weighed, 7; deficient, 7. Series II, 7, 8, and 8, respectively.

[2] Number of animals: ad libitum, 3; pair-weighed, 3; deficient, 4.

[3] Standard error.

Specific activities of the liver lipids were in all cases greatest in the pair-weighed controls. These animals also incorporated the largest proportions of the injected C^{14} into liver lipids. The data suggest that in the ad libitum-fed controls, the rate of incorporation of injected acetate-C^{14} into fat might be less rapid because of an already generous supply of acetate from food and endogenous sources of body stores. On the other

hand, the deficient animals would seem to have lost the ability of the underfed controls to use the injected acetate rapidly and efficiently as a precursor for the synthesis of liver lipid. The deficient and underfed control animals possibly do not have the endogenous sources of acetate.

TABLE 2

Incorporation of acetate-2-C^{14} into liver and carcass lipids and liver cholesterol

	SERIES I [1]		SERIES II	
	Specific activity [2]	% injected dose	Specific activity	% injected dose
Total lipids:				
Ad libitum	159 ± 21	1.69 ± 0.13	331 ± 70	2.41 ± 0.48
Pair-weighed	653 ± 115	5.30 ± 1.00	843 ± 72	5.29 ± 0.68
Deficient	164 ± 36	0.96 ± 0.27	401 ± 96	2.39 ± 0.78
Total cholesterol:				
Ad libitum	67 ± 17	0.034 ± 0.009	50 ± 3	0.022 ± 0.001
Pair-weighed	275 ± 53	0.086 ± 0.015	331 ± 69	0.108 ± 0.007
Deficient	119 ± 27	0.038 ± 0.013	125 ± 29	0.044 ± 0.009
Free cholesterol:				
Ad libitum	57 ± 10	0.023 ± 0.005	65 ± 4	0.025 ± 0.001
Pair-weighed	301 ± 86	0.071 ± 0.014	410 ± 84	0.097 ± 0.015
Deficient	94 ± 9	0.023 ± 0.003	146 ± 30	0.045 ± 0.010
Carcass lipids:				
Ad libitum			13	10.2
Pair-weighed			48	15.8
Deficient			47	14.6

[1] Series I, dosage 3.96×10^6 cpm; series II, 5.60×10^6 cpm.

[2] Measured in counts per minute per milligram.

Total liver cholesterol percentages were not very different in the ad libitum-fed, pair-weighed, and deficient rats. The smaller liver sizes of the pair-weighed and deficient animals were, however, reflected in smaller absolute amounts of liver cholesterol.

Specific activities of liver cholesterol were highest in the underfed control rats, and lowest in the ad libitum controls. The percentage of the injected dose of acetate-2-C^{14} incorporated into cholesterol might, as in the case of the total liver

lipid, be considered to be low in the ad libitum-fed rats because extra acetate was not needed as a percursor in cholesterol synthesis. Likewise, activity might be considered to be high in the underfed controls because they needed, and were able to use, acetate in the synthesis of cholesterol. Again, the data suggest that the biotin-deficient rats might be making less efficient use of the two-carbon moiety in the synthesis of cholesterol than were their underfed controls. Since a large proportion of the liver cholesterol is "free" in rats fed, as were all of these, nearly cholesterol-free diets, it is interesting that the specific activities of the free cholesterol were within the same ranges as those of the total liver cholesterol.

TABLE 3

Glycogen in the liver and incorporation of acetate-2-C^{14}

SERIES		% MOIST WT.	MG IN LIVER	CPM/MG	% INJECTED DOSE
I	Ad libitum	6.0 ± 0.43	325	160 ± 23	2.08 ± 0.41
	Pair-weighed	6.8 ± 0.40	421	93 ± 23	0.91 ± 0.21
	Deficient	3.9 ± 0.55	217	243 ± 28	1.12 ± 0.08
II	Ad libitum	5.7 ± 0.38	659	167 ± 29	1.90 ± 0.18
	Pair-weighed	6.3 ± 0.69	483	95 ± 26	0.88 ± 0.25
	Deficient	5.5 ± 0.87	438	191 ± 40	1.35 ± 0.21

The specific activity of the carcass fat was definitely greater in the underfed controls and the deficient rats than in the ad libitum controls. Differences were more than great enough to balance absolute differences in amount of carcass fat. Here, however, the incorporation of acetate into the fat of the underfed control rats was not significantly greater than that of the deficient rats. Also, the lipid synthesized was probably chiefly glyceride.

The concentration and amounts of liver glycogen were significantly lower in the biotin-deficient than in the pair-weighed rats of series I (table 3). In the 10-week rats of series II, differences between groups were smaller than in the 7-week rats. In both series, the specific activity of the liver

glycogen was at least twice as high in the deficient as in the pair-weighed rats. Mean values were higher than those for specific activities of glycogen in the ad libitum-fed animals, but in this case the ranges were such as to make the differences of doubtful significance.

In general, the measurement of activity would indicate that oxidation to carbon dioxide and synthesis of glycogen seem to be the preferred pathways for use of the acetate moiety in the biotin-deficient rat. In contrast, synthesis of liver lipid and of cholesterol seem to be the preferred pathway for acetate in the underfed but otherwise normal rat.

Since the pair-weighed animals were killed 6 hours after beginning to eat, they must have been well past the period of stimulation of lipid synthesis from acetate described by Van Bruggen et al. ('55, '57) in rats which had been fasted and then re-fed just before injection with labeled acetate. Also, in the present study, the ad libitum-fed controls and the biotin-deficient rats, as well as the pair-weighed rats, were fasted and then re-fed for 6 hours previous to autopsy. The greater synthesis of lipid and cholesterol by the pair-weighed animals would seem, therefore, to be a true reaction to previous restriction of food intake. Further work will be required to determine whether the failure of the biotin-deficient rat to synthesize lipid at the same rate as his control, with approximately the same food intake, was a direct result of the deficiency or whether it was due to the fact that the deficient animals ate their diet bit by bit throughout the 24 hours, while the pair-weighed rats usually ate their day's food supply within about 4 hours. That biotin deficiency actually does inhibit glyceride, phospholipid, and cholesterol synthesis is suggested not only by the failure of the biotin-deficient animals to show the same extra C^{14} activity in liver lipids as their pair-weighed controls, but also by the evidence that they use acetate preferentially for glycogen synthesis. A possibility which also deserves mention is that, in biotin deficiency, the rate of synthesis of certain specific fatty acids (possibly oleic) may be sufficiently low to decrease the animal's capacity to build up phospholipid and

cholesterol ester molecules. This might result, under stress of cholesterol feeding, in the lowered capacity for mobilization and storage of cholesterol ester previously noted in biotin-deficient rats.

SUMMARY

Biotin-deficient, pair-weighed control, and ad libitum-fed control rats were injected with acetate-2-C^{14}, one series after 7 and another after 10 weeks on the diet. During a 4-hour period, the biotin-deficient rats were found to excrete the largest proportion of the injected $C^{14}O_2$, with the highest specific activity. The pair-weighed controls retained the largest proportion of the injected C^{14}. The concentration of liver lipids was slightly higher in the pair-weighed rats than in the other two groups. In all cases, the specific activities of the liver lipids and of free and total cholesterol were much the highest in the underfed controls of the pair-weighed groups, as were the percentages of the injected C^{14} found in the liver lipids. Carcass fat also had the highest specific activity (counts per minute per milligram) in the pair-weighed groups, but in this case the differences between the pair-weighed and deficient groups were small. Liver glycogen, in contrast, showed the greatest activity in the deficient animals. The extent to which the data suggest impairment or alteration in the metabolism of acetate in the biotin-deficient rat is discussed.

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SOME EFFECTS OF *DL*-METHIONINE AND GLYCOCYAMINE ON GROWTH AND NITROGEN RETENTION IN RATS [1]

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INTRODUCTION

Dietary methionine, in small quantities, has been shown to augment the rate of growth in animals (Allison et al., '47) on a 12% casein diet. However, large quantities of methionine produce toxic reactions such as inhibition of growth (Brown and Allison, '48; van Pilsum and Berg, '50), severe weight loss (Earl et al., '42; Reisen et al., '46) and hemosiderin in the spleen (Cohen and Berg, '51).

Methionine is an essential amino acid utilized not only in the synthesis of protein, but as a methylating agent (du Vigneaud et al., '41). An inadequate supply of methyl acceptor substances such as glycocyamine (guanidoacetic acid) may account for the toxicity of an excess of methionine.

Glycocyamine, formed from the amino acids arginine and glycine (Borsook and Dubnoff, '41, '47) is distributed throughout animal tissues and is a normal constituent of the urine (Jaffe, '06; Weber, '36; Bodansky et al., '37). An excess of glyocoyamine in the diet also inhibits growth (du Vigneaud et al., '40) and produces toxic effects, such as, the production of fatty livers (Stetten, '42), hemorrhage in the kindneys

[1] A preliminary report was presented before the American Institute of Nutrition, Atlantic City, April, 1954 (Federation Proc. *13:* 450, 1954).

(Stetten and Grail, '42) and genito-urinary effects (Jaffe, '06; Dorner, '07; Fallis and Lam, '52). Excess glycocyamine in the diet may create an increased demand for methionine and, by draining available stores, cause a methionine deficiency.

Conflicting results have been reported when both methionine and glycocyamine are present in the diet. Hoberman et al. ('48), Borsook et al. ('51), Borsook and Borsook ('51) note that the combination of methionine and glycocyamine is toxic, while McKittrick ('47) and du Vigneaud et al. ('40) record that it is not.

This paper presents a summary of observations from experiments set up to determine more adequately the interrelations and the effect of methionine, of glycocyamine, and of their combinations, upon the growth, food efficiency and nitrogen retention of immature animals.

PROCEDURES AND METHODS

Normal male Wistar rats, weighing approximately 30 gm each were used in these investigations. The animals were divided into groups according to body weight so that the average initial weight of all groups was the same.

A purified diet was used, prepared by the method described by Roth and Allison ('50). The basal diet had the following composition: vitamin test casein [2] 12, sucrose 15.4, dextrin 20.2, glucose 22.2, Wesson ('32) salt mix 1.7, hydrogenated fat [3] 25.2 and agar 3.3%. Each 100 gm of dry diet also contained cod liver oil 1.5 ml and the vitamins in milligrams: calcium pantothenate, niacin and *p*-aminobenzoic acid 9.6 each, folic acid, 2-methyl, 1, 4-napthoquinone and biotin 0.048 each, thiamine 0.48, ascorbic acid 0.96, pyridoxine·HCl 0.385, riboflavin 0.768, inositol 24, choline 240 and α-tocopherol 500. Water was offered ad libitum to all animals.

DL-Methionine and glycocyamine were added at various levels to the basal diet as given in the appropriate figures. Food intake was recorded daily for each animal. All animals

[2] Labco.

[3] Crisco.

were placed upon the basal 12% casein diet for 4 days before the experiments were begun.

In the screening investigation, ad libitum feeding was used. The pair-feeding technique was utilized in the nitrogen balance study, so that all animals were fed almost identical quantities of food. Each animal was housed separately in a metabolism cage, the wire bottom of which permitted separation of feces from urine.

The urine was collected in Erlenmeyer flasks containing 2 ml of 0.1 N HCl. Five-day collections were pooled for analysis. Fecal collections were softened with water containing sulphuric acid, partially digested to a homogeneous emulsion with sulphuric acid and diluted with water. Total nitrogen was determined by a micro-Kjeldahl-Nesslerization method. Urea and ammonia nitrogen were analysed by the urease, direct Nesslerization method. Fisher's "t" table ('50) was used in appraising significance of differences between the means.

RESULTS

The data in figure 1 (left) illustrate weight gain over a period of 25 days in weanling rats fed a 12% casein diet, ad libitum, to which various amounts of glycocyamine and DL-methionine had been added. Each point in the figure represents an average obtained from 10 rats. The triangles illustrate the effect of adding 1, 2, 3 and 4% of glycocyamine to the diet. The maximum reduction in growth was reached upon the addition of 2% of glycocyamine ($p = 0.005$); the addition of 3 and 4% of glycocyamine showed no further change in growth.

The circles illustrate data obtained while feeding rats the 12% casein diet supplemented with 1% of DL-methionine, and 1% of DL-methionine with additions of 1, 2, 3 and 4% of glycocyamine. It is well known that the addition of small amounts of methionine to a low-protein diet such as 12% casein increases weight gain and food efficiency of growing animals. The addition of 1% of glycocyamine with 1% of DL-methionine, however, increased growth still further ($p = 0.02$) above that

observed with the amino acid alone. Further increments of glycocyamine tended to reduce growth below this high value, but the growth remained significantly above that of the control.

The crosses illustrate data obtained while feeding 12% of casein supplemented with 2% of DL-methionine and with 2% of DL-methionine plus 1, 2, 3 and 4% of glycocyamine. This

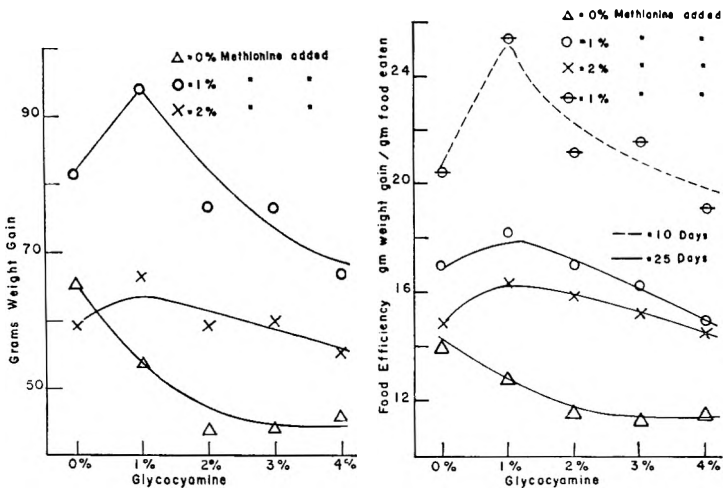

Fig. 1 (left) Weight gained by ad libitum feeding 12% casein with 0, 1, 2, 3 or 4% glycocyamine and 0% (triangles), 1% (circles) or 2 % (crosses) methionine. Each symbol represents values obtained on 10 rats fed ad libitum for 25 days.

Fig. 1 (right) Food efficiency obtained by ad libitum feeding 12% casein with 0, 1, 2, 3 or 4% glycocyamine and 0% (triangles), 1% (circles) or 2% (crosses) methionine. Each symbol represents values obtained on 10 rats fed ad libitum for 25 days (solid line), or for 10 days (broken line).

amount of amino acid is in excess of the optimum for growth and did not increase weight gain above that of the controls fed 12% of casein; however, it did overcome significantly ($p = 0.005$) the marked reduction in weight gain by animals fed 1, 2, 3 or 4% of glycocyamine.

The data in figure 1 (right) show that the food efficiencies parallel gains in weight for animals fed the 12% casein diet to which various amounts of glycocyamine and methionine have been added. The food efficiency, that is, grams in body

weight per gram of food eaten, of the glycocyamine groups, the 1% methionine groups and the 2% methionine groups again were of the order of magnitude which parallel weight gain. Rats fed the mixture of 1% DL-methionine and 1% glycocyamine showed increased food efficiency above all other diets tested, particularly during the early growth period.

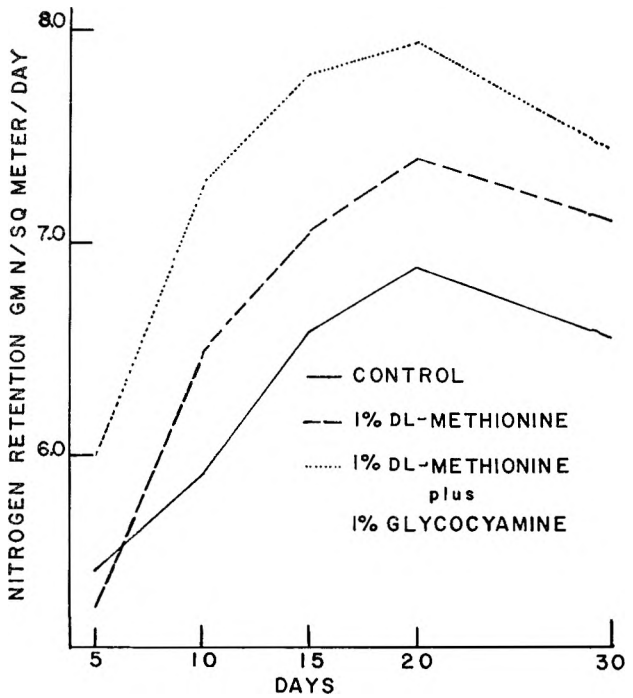

Fig. 2 Average nitrogen retention for 12 pair-fed rats in each group, 12% casein control (solid line), 1% DL-methionine (dash line) and 1% DL-methionine plus 1% glycocyamine (dotted line).

In order to determine the effect of glycocyamine and DL-methionine on nitrogen balance, three groups of rats (12 in each group) were pair-fed in terms of casein in the diet. One group received the unsupplemented diet, the second group was fed the diet plus 1% of methionine, while the third group received the diet plus 1% of DL-methionine and 1% of glycocyamine. The data in figure 2 demonstrate that adding

methionine increased nitrogen retention above that of the control, but that when 1% quantities of DL-methionine and glycocyamine were added to the diet, nitrogen retention was significantly increased above that of the control and of the group receiving 1% of DL-methionine alone.

Weight gains of the three pair-fed groups over a 25-day growing period were in the same direction as those graphically represented in figure 1, being 62 gm in the controls, 69 in those rats fed 1% of DL-methionine and 75 gm in those fed 1% DL-methionine plus 1% of glycocyamine. The lower weight gain in the pair-fed animals over those fed ad libitum may be the result of slight food restriction by the pair-fed groups. The significance of the differences was, therefore, lower in this experiment.

The urea plus ammonia nitrogen excretions observed in the foregoing experiment (nitrogen retention) were similar to those for animals fed either the 12% casein diet, the basal diet with the addition of both DL-methionine (1%) and glycocyamine (1%), or the basal diet with the addition of only DL-methionine (1%).

DISCUSSION

These experiments demonstrate that excess glycocyamine (guanidoacetic acid) in the diet reduced the rate of growth of young rats. The results suggest further that the metabolic pathways involved in the reduction of growth rate become saturated with 2% of glycocyamine in the diet, since 3% and 4% of glycocyamine show no further reduction in growth. Stetten and Grail ('42), du Vigneaud et al. ('40) and Borsook and Borsook ('51) have suggested that glycocyamine may drain the stores of available labile methyl groups, thus causing a methionine deficiency.

The present experiments show that when the methionine-deficient 12% casein diet is supplemented with 1% of DL-methionine, increased gain in weight occurs. The greater increase in weight in the presence of 1% of DL-methionine plus 1% of

glycocyamine suggests that such greater growth may be the result, in part, of the better utilization of available methionine when glycocyamine is present, and possibly, of glycocyamine itself. This seems to be particularly evident when the animal is under stress. The present experiments demonstrate that the addition of methionine to glycocyamine in suitable concentrations overcomes to a large degree the growth inhibition caused by glycocyamine feeding.

It is probable that DL-methionine added in larger than optimum amounts to the diet affects metabolic mechanisms not involving glycocyamine. This was indicated in our own results and in those of McKittrick ('47), who obtained slightly increased but not optimum chick growth when 0.58% of glycocyamine was added to a diet containing excess (1.5%) of methionine.

Cohen and Berg ('51) reported that the addition of 1.8% of methionine to the diet resulted in retardation of growth. The addition of a molecularly equivalent quantity of glycocyamine to the diet did not overcome this retardation, whereas molecularly equivalent additions of the glycocyamine precursors, arginine and glycine, did. Similar results were reported by Hardin and Hove ('51) with weanling rats on a 10% casein diet augmented with 2% methionine. As a result of the addition of arginine and glycine to the diet, metabolites other than glycocyamine may be produced. The addition of arginine to an excessive (4.8%) methionine diet (12% casein) reduced the loss in weight somewhat (Brown and Allison, '48) but the addition of glycine to this arginine-methionine diet almost overcame the weight loss associated with excess methionine and increased nitrogen balance (Roth and Allison, '49; Roth, Allison and Milch, '50). It is probable, therefore, that less than the theoretically possible quantity of glycocyamine is formed, whereas the addition of molecularly equivalent glycocyamine may be excessive.

The data in these experiments demonstrate that weight gains are correlated in part with food efficiencies (that is, grams gain in body weight per gram of food eaten) and with

food intake. In general, rats eat less on diets that are poorly balanced for their nutrition, and at the same time there is a reduction in food efficiency. The increase in food efficiency for rats fed the mixture of 1% of DL-methionine and 1% of glycocyamine above all the other diets tested is particularly evident during the early growth period. It would seem, therefore, that when the amount of glycocyamine added to the diet can metabolically balance the methionine present, there is improved growth and resistance to stress (Baron and Allison, '54). When the balance is upset by an excess of either substance, there may result a lack of growth and lowered food efficiency.

In the present experiments there was an increase in nitrogen retention when rats were pair-fed a 12% casein diet to which was added 1% of methionine and 1% of glycocyamine as compared with that of animals on control or on 1% methionine diets. It has been shown that the addition of nitrogen in the form of other nitrogen-containing substances, such as glycine, to a 12% casein diet, produce no significant changes in growth rate or nitrogen balance (Roth and Allison, '49; Hawkes, Henderson and Elvehjem, '49).

The excretion of urea plus ammonia is not altered significantly by any of the diets. This can be interpreted to mean that amino acid catabolism is not affected.

It may therefore be stated that the addition of 1% of methionine and 1% of glycocyamine to the low-protein 12% casein diet is associated with an increase in nitrogen retention, an increase in food efficiency and a greater gain in weight, all indicative of nitrogen utilization greater than that obtained by methionine addition alone.

SUMMARY

1. Nitrogen retention and growth by weanling rats pair-fed a 12% casein diet containing 1% of methionine and 1% of glycocyamine was significantly increased above that of the controls fed 12% casein alone or with a 1% DL-methionine

supplement. The excretion of urea plus ammonia nitrogen was approximately the same for all groups.

2. The inclusion of 1% of methionine in a 12% casein diet fed ad libitum increased the growth rate, but the addition of 1% glycocyamine increased growth rate (and food efficiency) even further. The use of 1% of DL-methionine with 2, 3 or 4% glycocyamine reduced growth rate below this high value, although it remained above that of the controls.

3. Although the rate of growth did not increase above that of the controls when 2% of methionine was added to the diet, this amount of methionine did overcome the reduction in growth rate noted when glycocyamine alone was added in 1, 2, 3 or 4% quantities to the basal diet.

4. Food efficiencies were found to parallel weight gains on the various diets. Animals fed the basal diet with 1% of DL-methionine and 1% of glycocyamine showed the highest food efficiency.

5. These observations suggest that methionine not only can overcome the toxic effects of glycocyamine, but that if both are added in metabolically balanced quantities to a 12% casein diet, there is a significant increase in weight gain, food efficiency and nitrogen retention over that of any of the diets tested.

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INFLUENCE OF HIGH-FAT DIETS ON GROWTH AND DEVELOPMENT OF OBESITY IN THE ALBINO RAT [1]

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INTRODUCTION

While studying the possible influence of high-fat intakes on the genesis of degenerative diseases in the rat, several experiments were carried out to compare the growth-promoting and obesity-producing properties of some of the animal and vegetable fats.

The differences in nutritive value of various oils and fats have been the subject of several reviews and studies, and generally special attention was given to the possible differences between butterfat and other oils and fats (Cowgill, '45; Smith, '48; Thomasson, '55; Dryden et al., '56). The obesity-producing properties of edible oils and fats seem to have been explored to a much lesser degree and, until recently, it was considered quite difficult to produce marked obesity in the rat by dietary means only. Hypothalamic lesions (Brobeck, '43; Kennedy, '51), or forced feeding (Wissler et al., '49) have served to induce obesity in the rat.

After some preliminary experimenting, a high-fat diet was developed at this laboratory which, when fed for long enough periods, resulted in weight gains of over 1000 gm. The present investigation deals with the effect of some animal and vegetable fats on the growth of rats when these fats are fed in high concentration for long periods.

[1] This work was partially supported by a grant from the Life Insurance Medical Research Fund, and funds granted by the American Dairy Association.

EXPERIMENTAL

Seven different experiments involving more than 600 rats were conducted, using from 12 to 40 animals per group. The rats were young male albinos of the Sprague-Dawley strain. Their initial average weight varied from one series to another, ranging from 35 to 95 gm. The duration of the experiments varied from 6 weeks to two years.

The animals were housed in individual, metal, screen-bottom cages, in an air-conditioned room at a temperature of 70°F. and 40 to 45% relative humidity. The following fats were tested: lard,[2] butter,[3] vegetable shortening,[4] margarine,[5] corn oil,[6] coconut oil, and cottonseed oil. Three to 6 of these fats were included in each series; each series also included a high-carbohydrate control group of rats.

The high-carbohydrate control diet had the following percentage composition: casein 20,[7] corn oil 5, animal and vegetable shortening 5,[8] dextrin 65.6, salt mixture 4,[9] methionine 0.2, and choline 0.2. The vitamin levels usually used in rat experiments in this laboratory were doubled to prevent possible vitamin deficiencies, which would result from an expected lower intake of these caloric-rich diets. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 10.0; *l*-inositol, 20; *p*-aminobenzoic acid, 20.0; folic acid, 0.1; biotin, 0.1; menadione, 1.0; and α-tocopherol acetate, 10.0. Vitamins A and D were supplied in the form of halibut oil and a vitamin D_2 preparation.[10]

[2] Wilson's.

[3] Butterfat was used in series 1 and 2; Clearbrook brand butter was used in the remaining 5 series. The basal diet was adjusted accordingly to account for the difference in nutrient content.

[4] Crisco.

[5] Wilson's Golden Brand.

[6] Mazola.

[7] Borden's B_3F.

[8] Swift's shortening from animal and vegetable fats.

[9] Hegsted et al., '41.

[10] Drisdol, Winthrop-Stearns, Inc.

The high-fat or oil diets were prepared by replacing the carbohydrate and fat of the control diet by the specific fat in question. The Caloric density of the control diet in Calories per gram was 4.3, while that of the high-fat diet was 6.8. The total fat content of the high-fat diet was 61.6%. To prevent the separation of oils from the diet mix, they were first emulsified with 16 ml of polyoxyethylene (8) monostearate [11] and 200 ml of water per kilogram of diet and then mixed with the solid components. The diets were prepared in batches of 4 to 8 kg, which were stored in a cold room until used.

Food and water were offered ad libitum and the weights of animals were determined at weekly intervals. In one series, food intake was also measured. The animals which died during the experiment or were sacrificed at the end of the respective experimental periods were examined for gross pathology, and in some series, aortas, heart, livers and kidneys were taken for histological examination. On some animals, total cholesterol content of the blood serum was determined, using the Bloor ('28) method.

RESULTS

Differences in growth-promoting properties of the different fats made their first appearance within three weeks. In most series, the groups receiving liquid vegetable oils in their diet (e.g. corn oil, coconut oil, cottonseed oil) showed significantly smaller weight gains than those fed fats (e.g. lard, butter, margarine). The growth rate of the high-carbohydrate control group fell between these two fat-fed groups. These differences between the oils and fats persisted through the whole testing period. Comparing the individual vegetable oils, the groups on the corn oil diet gained the most weight, followed by the group on cottonseed oil and coconut oil. Of the solid fats tested, lard, Crisco and butter seemed to promote the greatest weight gains during the first two months of the experiment, with the margarine group generally trailing a little behind. In the later stages of the experiments,

[11] Atlas Powder Co., Myrj 45.

TABLE 1

Influence of dietary fats on weight gains of rats after (a) three weeks and (b) two months of the experiment

	DIETARY FAT	SERIES NUMBER 1	2	3	4	5	6	7
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
3 weeks	Corn oil	50 ± 6.0 [1]	130 ± 6.7 [2]	53 ± 2.3 [3]	84 ± 4.1	..	..	64 ± 8.1
	Coconut oil	70 ± 6.3	111 ± 5.6	..	..	..	..	53 ± 6.3
	Cottonseed oil	47 ± 5.3	..	..	..	..	..	..
	Lard	127 ± 6.4	163 ± 2.8	68 ± 2.7	97 ± 3.8	..	91 ± 7.3	124 ± 9.0
	Butterfat	123 ± 6.9	161 ± 5.1	69 ± 2.5	92 ± 4.4	100 ± 3.3	101 ± 9.0	..
	Margarine	..	..	60 ± 1.7	92 ± 4.8	96 ± 2.9	97 ± 6.2	..
	Crisco	135 ± 4.4	..	..	..	101 ± 3.6	..	..
	Controls	96 ± 3.8	150 ± 5.0	65 ± 2.4	95 ± 2.8	83 ± 1.6	105 ± 5.7	84 ± 6.3
2 months	Corn oil	271 ± 8.9	258 ± 3.9	250 ± 5.1 [4]	200 ± 7.1 [5]	..	..	239 ± 18.5
	Coconut oil	247 ± 8.4	240 ± 2.4	..	..	..	..	211 ± 10.6
	Cottonseed oil	260 ± 9.2	..	..	..	..	..	..
	Lard	314 ± 6.7	298 ± 3.8	287 ± 2.3	222 ± 5.7	..	244 ± 4.8 [4]	280 ± 14.1
	Butterfat	311 ± 10.2	281 ± 2.8	270 ± 2.5	195 ± 5.3	297 ± 6.5	261 ± 6.1	..
	Margarine	..	..	270 ± 5.6	210 ± 5.5	266 ± 3.7	253 ± 5.9	..
	Crisco	284 ± 6.3	..	..	..	301 ± 5.9	..	..
	Controls	218 ± 6.2	260 ± 5.9	267 ± 3.0	185 ± 2.3	295 ± 6.1	249 ± 11.5	265 ± 16.5

[1] Mean ± standard error of the mean.
[2] Weight gains for 25 days.
[3] Weight gains for 14 days.
[4] Weight gains for 55 days.
[5] Weight gains for 42 days.

the groups fed lard and Crisco showed the greatest weight gain, followed by the butter and margarine groups. The butter groups in series 4 and the lard groups in series 6 for some unknown reason showed lower weight gains than the corresponding groups in other series and the data of these groups were not considered in the final evaluation. The weight gains on the same diet and in comparable time periods differed slightly from one series to another, and were probably due to the differences in the initial body weights.

TABLE 2

Final weight gains in the long-term experiments

DIETARY FAT	SERIES NUMBER		
	5 [1]	6 [2]	7 [3]
	gm	*gm*	*gm*
Corn oil	..	..	769 ± 43
Coconut oil	..	..	652 ± 51
Lard	..	641 ± 10	1076 ± 136
Butterfat	666 ± 21 [4]	709 ± 68	..
Margarine	576 ± 22	704 ± 62	..
Crisco	707 ± 32	..	..
Controls	504 ± 14	589 ± 50	622 ± 54

[1] During 258 days.
[2] During 475 days.
[3] During 575 days.
[4] Mean ± standard error of the mean.

The weight gains of some animals were spectacular. Within 280 days two animals on the lard diet in series 7 and three animals on the Crisco diet in series 5 surpassed the weight of 900 gm. After 11 months, the animals on the lard diet in series 7 started to reach the 1000 gm limit. Ultimately, 80% of the animals in this group reached this weight, the heaviest animal weighing 1450 gm. Of the other groups, three animals on the corn oil diet in series 7 and one animal on the butter diet in series 6 reached the 1000 gm weight limit. The series 5 experiment was discontinued after 285 days.

Mortality seemed to be related to the average initial weight of the animals. In series 6, in which the initial weight was

about 35 gm, 60 to 90% of the animals died before the 600th day of the experiment; most of them at the very end of the experiment. In series 7, where the average initial weight was 95 gm, only 30 to 55% of the animals died before the 600th day; the highest mortality rate was that of the lard group, the lowest that of the control. In series 5, the food intake was measured for 285 days and the food efficiency (grams of food eaten per gram of weight gain) calculated. Over the whole experimental period, the animals fed the Crisco diet showed the highest food efficiency, namely 4.8 gm of food per gram of weight gain, followed by butter 5.4 gm, margarine 5.7 gm, and the high-carbohydrate control 10.3 gm.

Aortas, coronary arteries, livers and kidneys of animals in the longer term series 5, 6, and 7 were examined histologically. In series 5, no pathological changes in these organs could be detected. In the other two series, a few cases of shedding of aortic intima or of thickened fibrous subendothelium were found in some groups, including the controls. The animals on high-fat diets showed some fatty infiltration of the liver, especially in series 7, but no cases of distinct fibrosis or sclerosis were seen. In the kidneys, some lesions in convoluted tubules and cortices were noted in all groups. The renal arteries, however, were found to be without any unusual changes.

In series 7, total cholesterol in blood serum was determined on several animals in each group. The range of cholesterol values for the lard group was 56 to 216 mg%, for the coconut oil group 50 to 135 mg%, for the corn oil group 59 to 186 mg%, and for the high-carbohydrate control group 57 to 157 mg%.

DISCUSSION

The results of this investigation indicate that the solid fats (lard, butter, Crisco or margarine), when fed in very high concentrations, promote the growth of young rats more effectively than vegetable oils (e.g. corn oil, coconut oil or cottonseed oil). The differences in the growth-promoting values of vegetable and animal fats when fed at high levels were observed also by Thomasson ('55). This author com-

pared the growth-promoting values of 20 oils and fats at different levels and found that at higher levels of fats in the diet, lard and butter lead to higher gains than the comparable diets containing vegetable oils. Boutwell et al. ('43), testing the growth of young rats, fed vegetable oils, lard and butter at a level of 28%, and also found that the animals fed vegetable oils gained less weight than those in the lard and butterfat groups. More recently, Dryden et al. ('56) reported that the differences in weight gains between the animals receiving either corn oil or butterfat are more pronounced with the increase of fat level in the diet. The differences in growth-promoting properties of the tested fats could be at least partially explained by the observation of Barki et al. ('50), who reported that the levels of fat necessary for optimum growth differ from one fat to another.

Coconut oil was found in our experiments to have the lowest growth-promoting value of the tested fats. Other reports in the literature (Thomasson, '55; Barki et al., '50) also indicate that coconut oil, when fed at high levels, does not support the growth of animals as well as the other fats. According to Channon et al. ('37) lauric acid, one of the main fatty acid components of coconut oil, is incorporated very slowly into the body fat, which could account partly for the lower weight gains obtained with this particular oil.

Concerning the development of obesity by dietary means only and the differences between individual fats, only a few reports were found in the literature and these were reviewed by Mickelsen et al. ('55). These authors were also able to produce extremely obese rats similar to ours, using high levels of Crisco. Our results indicate that there is a significant difference between the fats in the production of obesity. Of the fats tested, lard and Crisco seem to be the ones producing obesity most readily, followed by butter and margarine, then by corn and coconut oils. The fact that some animals on the corn oil diet ultimately reached the arbitrary weight limit of over 1000 gm, indicates that the differences in obesity-producing properties of fats may be mainly a reflection of differences

in transformation of dietary fat to body fat. The results of carcass analysis reported by Dryden et al. ('56) also indicate that, in many cases, the differences in weight gains can really represent differences in amounts of stored fat. A detailed study of individual fats and their influence on body composition will be necessary to answer this question.

Although it is known that the rat is rather resistant to arterial lesions, the relative lack of histological evidence of arterial damage in severe conditions of this experiment is interesting. After the animals had been on high-fat diets for 285 days, no pathological changes were found in the aorta or heart. After about two years on experiment, cases in some groups, including the controls, were found showing some changes in aortic subendothelium. However, no special connection between the type of fat and the degree and extent of lesion was found. Moderate to heavy deposition of fat in livers of all high-fat groups was noticed, and again no significant difference between the groups could be detected. The kidneys of some animals showed mild swelling of tubules and capillary congestion in the cortex. No systematic influence of fat or oils on kidney damage could be found.

It is possible that the animals in these experiments failed to develop notable arterial pathology despite the prolonged intake of high-fat diets, the development of marked obesity and, in some cases, pronounced hypercholesterolemia, because the percentage of protein Calories was maintained at a high level and the vitamin intakes were abundant at all times.

SUMMARY

The growth-promoting and obesity-producing properties of certain animal and vegetable fats were studied in several rat experiments. The fats were fed at high concentration, contributing 81% of the Calories. The vegetable oils tested (corn oil, coconut oil, and cottonseed oil) did not promote growth in young rats as efficiently as lard, Crisco, margarine, or butter. With respect to the obesity-producing properties, lard and Crisco were most effective, then butter and mar-

garine, with corn oil and coconut oil showing the least effect. About 80% of the rats in the lard group reached a weight greater than 1000 gm. Some cases of fatty infiltration of the aortic subendothelium and deposition of fat in livers were noted. No special lesions in relation to any of the high-fat diets could be established.

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EFFECTS OF SHORT-TERM PANTOTHENIC ACID DEFICIENCY IN THE GROWING RAT [1]

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INTRODUCTION

Lack of pantothenic acid in the diet of growing rats has been reported to depress growth (Unna, '40; Barboriak et al., '57a), and to adversely affect some organs (Ashburn, '40; Dean and McKibbin, '46). As the metabolically active form of pantothenic acid – coenzyme A – participates in many important basic processes, such as acetylation, fat synthesis, and Krebs cycle (Novelli, '53), it would be of interest to know how a short-term deficiency of pantothenic acid during the important growth stage would affect the subsequent growth, well-being and pantothenic acid requirement of the animals. The results of such an investigation, as well as the effect of the length of deficiency period, are described in this paper.

EXPERIMENTAL

Weanling albino rats of the Sprague-Dawley strain, weighing initially about 50 gm were fed a pantothenic acid-deficient diet for three, 4, and 5 weeks. The diet was then supplemented with graded amounts of pantothenic acid.

For each three- and 5-week deficiency period, 30 male rats were used. After the respective depletion periods, the animals were divided into three groups, each consisting of 10 animals,

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and their diets supplemented with 0.3, 0.6, and 1.2 mg of calcium pantothenate per 100 gm diet.

In the 4-week deficiency period, 45 males were used. After depletion, they were divided into three groups and fed 0.2, 0.5, and 2.0 mg of pantothenate per 100 gm of diet. After 19 days, the group receiving 0.5 mg of pantothenate was subdivided; for 7 animals the vitamin level was increased to 0.8 mg per 100 gm of diet, and the remaining 8 animals continued on their original level of 0.5 mg of pantothenate. Ten animals receiving 10.0 mg of pantothenate per 100 gm of diet and not exposed to a deficiency period served as a control group.

The composition of the pantothenic acid-deficient diet in per cent was as follows: vitamin-free casein,[2] 25; salts IV,[3] 4; corn oil, 5; choline chloride, 0.2; cystine, 0.2; and sucrose, 65.6. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 10.0; *l*-inositol, 20; *p*-aminobenzoic acid, 20.0; folic acid, 0.1; biotin, 0.1; menadione, 1.0; and α-tocopherol acetate, 10.0. Vitamins A and D were supplied in the form of halibut oil and a vitamin D_2 preparation.[4] With respect to vitamin B_{12}, the relatively high casein content, 25%, employed in the present study, is believed to have been adequate to prevent any vitamin B_{12} deficiency. The animals that received pantothenic acid grew at a rate considered to be normal.

The animals were kept in individual metal, screen-bottom cages, in an air-conditioned room at 70°F and 40 to 45% relative humidity. Food and water were offered ad libitum. Weights of animals were recorded weekly for a 7-week period, following the supplementation of the deficient diet. The animals in the three-week and 5-week deficiency periods were then sacrificed, their adrenals weighed and, together with testes, preserved in buffered formaldehyde for histological

[2] Borden's Labco.

[3] Hegsted et al. ('41).

[4] Drisdol, Winthrop-Stearns, Inc.

examination. The animals in the 4-week deficiency period were subsequently used for a mating study (Barboriak et al., '57b).

RESULTS

The diminished growth rate, usually observed in animals fed a pantothenic acid-deficient diet, became apparent after two to three weeks. The weight of the deficient animals tended to stabilize between 80 and 100 gm body weight. The pantothenic acid supplements resulted in an intense growth response and the growth rate seemed to be related to the vitamin level of the diet. After the 5-week deficiency period, there was a time lag of about 10 days before the groups receiving 0.3 and 0.6 mg of pantothenate responded to the vitamin supplement. The growth rate of animals receiving the highest vitamin levels usually equaled or even surpassed that of the control group during the corresponding time intervals. For instance, the animals fed a pantothenic acid-deficient diet for 5 weeks and then supplemented with 1.2 mg of pantothenate per 100 gm of diet, gained an average of 196 gm during the 7 weeks following the deficiency period, as compared with 193 gm gained by the controls during the corresponding 7-week period.

External signs of pantothenic acid deficiency, such as thinning of the fur and porphyrine whiskers, appeared soon after the growth rate was reduced, and they tended to persist even after the vitamin supplements were fed.

Histological examination of the testes of the animals in the three-week deficiency groups did not reveal any significant pathological changes. The cell structure was normal and spermiogenesis abundant in most glands. In the 5-week deficiency group, the testes of animals receiving 0.3 mg of pantothenate showed breakdown of the germinal epithelium, degenerative changes in germ cell layers, and practically no sperm. The animals on the 0.6 mg pantothenate level still showed some damage in the germ cell layers; the spermiogenesis was, however, much improved. The testes of animals

TABLE 1

Average weight gains of animals fed pantothenic acid-deficient diets for three, 4, or 5 weeks and then supplemented with graded amounts of calcium pantothenate (CaP)

CaP/100 GM DIET	WEIGHT GAINS DURING				CaP/100 GM DIET	WEIGHT GAINS DURING	
	3-week deficiency	Subsequent 7 weeks	5-week deficiency	Subsequent 7 weeks		4-week deficiency	Subsequent 7 weeks
mg	*gm*	*gm*	*gm*	*gm*	*mg*	*gm*	*gm*
0.3	48 ± 4 [1]	72 ± 7	45 ± 4	96 ± 19	0.2	44 ± 3	65 ± 7
0.6	43 ± 2	157 ± 9	42 ± 4	177 ± 9	0.5	37 ± 4	96 ± 12
1.2	47 ± 4	207 ± 8	45 ± 3	196 ± 9	0.8	40 ± 3	143 ± 8
					2.0	43 ± 3	226 ± 5
10.0	106 ± 3 [2]	250 ± 12	185 [2] ± 4	193 ± 9	10.0	149 ± 4 [2]	218 ± 13

[1] Mean ± standard error of the mean.

[2] Gains of the control group during the corresponding vitamin supplemented period.

fed 1.2 mg of pantothenate were practically normal with very good production of mature spermatozoa.

The adrenal glands seemed to be equally affected regardless of the length of deficiency or the subsequent vitamin level fed. Several cases of congested and hemorrhagic zona reticularis were found in each experimental group. A rather low content of lipids in the zona fasciculata was observed in glands of animals fed 0.3 mg of pantothenate after 5-weeks of deficiency. The weight of adrenal glands per 100 gm of body weight, sometimes used as a criterion of pantothenic acid deficiency, was not related significantly to the length of deficiency or to the vitamin level.

DISCUSSION

The resumption of growth after the deficiency period indicates that even a relatively long period of severe pantothenic acid deficiency does not appreciably influence the growth capacity of the rat. These findings are in agreement with the general observation that refeeding of animals with a substance, the lack of which caused growth reduction, results in resumption of the growth process (Osborne and Mendel, '15; Clemmesen, '33; Schultze, '55). The data on weight gains suggest, however, that the deficiency period increases the subsequent vitamin requirement: weight gains comparable with those of the non-depleted controls were seen only with the highest vitamin supplements. In previous experiments (Barboriak et al., '57a), it was observed that a pantothenate level of 0.4 mg per 100 gm of diet insured a satisfactory growth rate. After a deficiency period, however, 1.2 to 2.0 mg were needed for comparable growth.

The persistence of external deficiency signs, observed also by Unna ('40), as well as the occurrence of adrenal lesions in animals fed large amounts of the vitamin, indicate that the damage caused by short-time severe pantothenic acid deficiency may be of a more permanent nature than one could surmise from the data on weight gains alone. It has been reported that the adrenal glands show pathological changes

quite early in pantothenic acid deficiency (Dean and McKibbin, '46). It is possible that more of the vitamin or a hormonal supplement is needed to repair the damage to the adrenal gland. Bean et al. ('55), reported that in human pantothenic acid deficiency, produced by combined feeding of a pantothenic acid-deficient diet and a pantothenic acid antimetabolite, pantothenic acid alone was not able to reverse the deficiency signs. Cortisone treatment was necessary to restore the health of the experimental subjects.

SUMMARY

The effect of three, 4, and 5 weeks of pantothenic acid deficiency on the subsequent development of young male rats during the following 7-week period was investigated. Lack of pantothenic acid resulted in arrest of growth in about two to three weeks. Supplementation of the deficient diet with calcium pantothenate led to renewed growth; the vitamin requirement for optimal growth was, however, increased. Despite the pantothenate supplements, external deficiency signs persisted and the adrenal glands of several animals showed typical changes encountered in pantothenic acid deficiency. It is concluded, therefore, that a short-term pantothenic acid deficiency in young growing rats may result in damage of a more permanent nature than believed previously.

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BIOLOGICAL AVAILABILITY OF LYSINE[1]

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The nutritive value of a protein is determined not only by its amino acid composition but also by the availability of the individual amino acids in the protein to the animal.

Several workers have used *in vitro* digestion procedures to determine the rate of release of amino acids from intact proteins. The results of these studies indicate that lysine is unique among the essential amino acids in that the rate, and in some cases the extent of its release from a protein, is decreased considerably when the dry protein is heated at high temperatures (Eldred and Rodney, '46; Pader et al., '48), autoclaved for several hours, (Hankes et al., '48) or heated with carbohydrates (Evans and Butts, '49), probably owing to the formation of enzyme resistant bonds involving the ε-NH_2 group. Similar observations have been made by Mauron et al. ('55) on the inactivation of lysine in spray-dried and roller-dried milk powder.

In view of these observations it is important to know the biological availability of the lysine in untreated and mildly treated proteins. The results of *in vitro* digestion studies do not provide a true index of availability as other factors such as a low rate of absorption of an amino acid or the failure of all essential amino acids to be available simultaneously (Melnick et al., '46; Cannon et al., '47; Geiger, '47) may limit the utilization of the amino acid in the animal body.

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Schweigert and Guthneck ('53) estimated the availability of lysine in proteins for the growth of adult protein-depleted rats. Kuiken and Lyman ('48) and Kuiken ('52) estimated the availability of lysine from a few proteins by measuring how much of the ingested lysine was excreted in the feces.

This paper deals with the estimation of availability of lysine from proteins using the growth of weanling rats as the index of availability. Included in the study were the proteins of two differently processed milk powders. The proteins of three common cereals, in which a low availability of lysine would aggravate the deficiency of this already limiting amino acid, were also examined. Availability was computed directly from the weight gains and also after correcting for differences in food consumption so that the two methods of calculation could be compared.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain weighing about 40 to 50 gm were used throughout the study. They were divided into similar groups of 6 animals each and were maintained in individual, suspended cages with screen bottoms. Food and water were given ad libitum and the animals were weighed weekly.

The percentage composition of the basal diet was: wheat gluten, 20; DL-methionine, 0.2; DL-threonine, 0.2; L-histidine·HCl, 0.2; DL-tryptophan, 0.05; Salts 4 (Hegsted et al., '41), 4; corn oil, 5; vitamin mixture in sucrose, 0.25; choline chloride, 0.15 and dextrin to make 100. The 0.25 gm of vitamin mixture contained in milligrams, thiamine·HCl, 0.5; riboflavin, 0.5; niacin, 2.5; calcium pantothenate, 2.0; pyridoxine, 0.25; biotin, 0.01; folic acid, 0.02; vitamin B_{12}, 0.002 and inositol, 10. Two drops of halibut liver oil fortified with vitamin E and vitamin K were administered orally each week. The only limiting factor in this diet was lysine. The protein supplements replaced equivalent quantities of dextrin.

The percentage availability of lysine from different proteins was estimated by comparing the growth responses obtained with these test materials as supplements to the basal diet with the responses obtained upon supplementing the diet with graded levels of crystalline lysine.

Differences in food consumption were taken into account in another method of computing availability. The percentage availability was calculated as above, from a comparison of the growth responses obtained with the test materials with those obtained with different levels of crystalline lysine, but in this case weight gains per 100 gm of food consumed were used, as described in a study of the biological availability of tryptophan (Gupta and Elvehjem, '57).

The lysine content of the proteins and of the feces was determined by the usual microbiological procedure with *L. mesenteroides* as the test organism. However, some modifications developed at the University of Wisconsin were used in the hydrolysis, in the preparation of the protein and cereal samples and in the actual analytical procedure (Hepburn et al., '57).

The food consumption of each rat was recorded. By placing the food cups in larger containers, losses from spillage were kept to a minimum.

Beef and pork samples were prepared as described by Gupta and Elvehjem ('57).

RESULTS

The growth responses obtained when graded levels of L-lysine (as hydrochloride) were added to the basal diet are shown in table 1.

Under the conditions of the experiment, the growth response was linear over the range of 0.27 to 0.77% of L-lysine. The basal diet contained 0.27% of lysine as determined by microbiological assay.

The growth responses obtained when sucrose was substituted for dextrin as the source of carbohydrate are also shown in table 1. The requirement for lysine as a percentage of the

diet was influenced by the type of dietary carbohydrate, being about 0.8% with dextrin and 0.9% with sucrose, in a diet containing moderate amounts of protein. Therefore, it is important, if weight gains are to be related to the level of an amino acid in the diet, to use similar dietary carbohydrates in the standard and experimental groups. However, if the weight gains are plotted against the total amino acid intake, the points fall almost in a straight line (fig. 1) irrespective of the nature of the dietary carbohydrate. This indicates that comparisons based on total amino acid intake are relatively

TABLE 1

Growth of rats with graded levels of lysine with dextrin and sucrose diets

L-LYSINE ADDED TO BASAL DIET	AVERAGE GAIN/2 WKS.		AVERAGE GAIN/ 100 GM FOOD	
	Sucrose	Dextrin	Sucrose	Dextrin
%	*gm*	*gm*	*gm*	*gm*
None	9.8	10.6	12.9	13.8
0.1	18.2	23.2	22.2	23.4
0.2	27.4	38.0	28.2	30.7
0.3	39.0	47.0	35.9	36.4
0.4	...	58.6	...	43.8
0.5	60.0	71.4	48.3	49.9
0.6	72.2	...	54.3	...
0.8	68.4	73.4	53.7	53.4

little influenced by the type of carbohydrate and probably by other factors that affect food consumption primarily.

Values for the availability of lysine from the different proteins which were fed at the 3% level (as determined by Kjeldahl nitrogen analysis) are shown in table 2. Besides being fed at the same level, all of the proteins except gelatin and the soybean protein provided nearly equal amounts of supplementary lysine. Therefore the comparisons were restricted to only a small portion of the standard curve.

It can be seen that lysine was available to the extent of 75 to 100% from the different proteins. In most cases the availability values were somewhat higher when the results were computed on the basis of equal food consumption than when weight gains were used directly.

Although the percentage availability of tryptophan (Gupta and Elvehjem, '57) and of isoleucine (Deshpande et al., '57) decreased somewhat when the level of supplementary amino acid was increased, values for the availability of lysine from roast beef fed at different levels with and without added L-lysine showed no decrease in availability with increased levels of lysine (table 3). The results obtained using weight

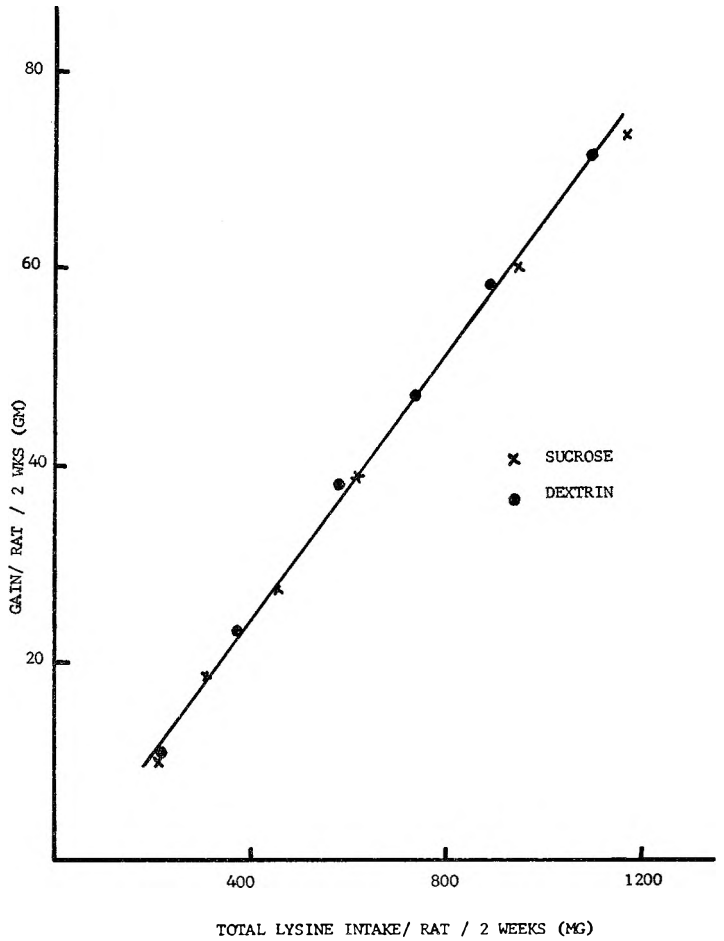

Fig. 1 Relationship between total lysine intake and weight gain of rats fed on diets containing sucrose or dextrin. Each point represents an average for 6 rats.

gains per unit of food consumed were again somewhat higher than those obtained by the direct method.

Values for the percentage availability of lysine from the proteins of three cereals and two differently processed milk powders which were fed at the same lysine level are presented in table 4. The lysine in roller-dried milk powder was much less available than that from spray-dried milk powder, a result which was expected from the *in vitro* digestion studies of Mauron et al. ('55). The values for the availability of

TABLE 2

Availability of lysine from different proteins

SUPPLEMENT ADDED	AMOUNT OF SUPPLEMENTARY LYSINE	AVAILABLE LYSINE AS READ FROM		AVAILABILITY AS CALCULATED FROM	
		A [1]	B	A	B
%	*gm/100 gm food*	*gm/100 gm*	*gm/100 gm*	%	%
Beef, 3	0.237	0.180	0.210	76	89
Casein, 3	0.221	0.223	0.230	101	104
Pork, 3	0.242	0.220	0.243	91	100
Gelatin, 3	0.111	0.082	0.105	74	94
Soybean protein, 3 [2]	0.146	0.150	0.172	103	118
Egg albumin, 3	0.175	0.162	0.150	93	91
Fish flour, 3 [3]	0.192	0.175	0.160	91	83

[1] A = Average gain/2 wks. B = Average gain/100 gm food.

[2] Drackett Assay protein, The Drackett Products Company, Cincinnati, Ohio.

[3] Viobin Corporation, Monticello, Illinois.

lysine from the proteins of the three cereals which are staple foods in many areas of the world, were distinctly different.

As the standard curve was based on the assumption that L-lysine added in crystalline form was fully utilized, it was important to verify this by determining the amount of ingested lysine which could be recovered from the feces of groups of animals fed two different levels of lysine. Further, the amounts of unavailable lysine in the feces of the rats of groups 1 and 3 (table 3) were also determined to see with which of the two methods of computation more consistent values would be obtained. These results are presented in table 5.

It can be seen that there is full utilization of the crystalline lysine and that only small amounts of lysine were recovered in the feces of the other two groups. This suggests that the values calculated using weight gain per unit of food con-

TABLE 3

Availability of lysine from beef protein fed at different levels

GR. NO.	BEEF ADDED	AMOUNT OF SUPPLEMENTARY LYSINE	AVAILABLE LYSINE AS READ FROM		AVAILABILITY AS CALCULATED FROM	
			A [1]	B	A	B
	%	*gm/100 gm food*	*gm/100 gm*	*gm/100 gm*	%	%
1	2	0.158	0.120	0.140	76	89
2	3	0.237	0.180	0.210	76	89
3	4	0.316	0.260	0.300	82	95
4	3 + 0.1 lysine	0.337	0.298	0.333	84	98
5	2 [2]	0.158	0.133	0.147	84	93
6	3 [2]	0.237	0.187	0.200	79	84

[1] A = Average gain/2 wks. B = Average gain/100 gm food.
[2] Results of a duplicate experiment.

TABLE 4

Availability of lysine from (a) cereals and (b) milk powders fed at the same lysine levels

SUPPLEMENT ADDED	AMOUNT OF SUPPLEMENTARY LYSINE	AVAILABLE LYSINE AS READ FROM		LYSINE AVAILABLE FROM	
		A [1]	B	A	B
%	*gm/100 gm food*	*gm/100 gm*	*gm/100 gm*	%	%
		1st experiment			
Spray-dried nonfat milk powder, 10	0.340	0.318	0.312	94	92
Roller-dried nonfat milk powder, 11.4	0.338	0.220	0.230	65	68
Corn, 50	0.128	0.075	0.075	58	58
Wheat flour, 53	0.136	0.108	0.095	79	70
Rice, 70	0.151	0.130	0.130	86	86
		2nd experiment			
Corn, 50	0.128	0.065	0.063	51	49
Wheat flour, 54.3	0.128	0.078	0.092	61	72
Rice, 59	0.128	0.103	0.110	81	87

[1] A = Average gain/2 wks. B = Average gain/100 gm food.
[2] Separate standard curves were run with each experiment.

TABLE 5

Unavailable lysine in some standard and sample groups

GROUP	TOTAL FOOD CONSUMED	TOTAL SUPPLEMENTARY LYSINE	TOTAL LYSINE IN FECES [1]	ENDOGENOUS LYSINE + UNAVAILABLE LYSINE FROM BASAL PROTEIN [2]	UNAVAILABLE SUPPLEMENTARY LYSINE	SUPPLEMENTARY LYSINE UNAVAILABLE
	gm	*gm*	*gm*	*gm*	*gm*	%
Basal	475	none	0.348	0.348		
Basal + 0.1% L-lysine	610	0.610	0.431	0.446	—0.15	—2.4
Basal + 0.3% L-lysine	844	2.53	0.580	0.717	—0.37	—1.5
Basal + 2% beef	596	0.942	0.508	0.437	.071	7.5
Basal + 4% beef	729	2.31	0.621	0.534	.087	3.8

[1] Determined by microbiological assay of a representative aliquot of the feces.

[2] Calculated as follows: $\frac{\text{Amount of lysine in feces of basal group}}{\text{Food consumed by basal group}} \times$ Food consumed by experimental group.

sumed, as shown in the last columns of tables 2, 3 and 4 represent a more accurate measure of availability.

DISCUSSION

The growth difference of about 60 gm, at the end of the two-week experimental period, between groups fed on the basal diet and those fed on the basal diet supplemented with the highest level of lysine, gave a very good range over which to measure availability. Also, as the response was almost linear for levels up to about 0.5% of added L-lysine, there were at least 6 points on the standard curve including that for the basal diet. This increased the accuracy of the standard curve when occasional groups were out of line.

That lysine was the only limiting factor in the basal diet is shown by the fact that a growth rate of about 35 gm/week, which is comparable to the growth rate of rats fed an adequate purified diet, was obtained when lysine was the only supplement provided. Also, since the weight gains with supplements of 0.3% lysine or 0.3% lysine plus 3% zein (protein devoid of lysine) were 27.2 and 27.7 gm/week, respectively, it seemed unlikely that an amino acid imbalance might have been created when the protein supplements were added to the lysine-deficient basal diet.

In calculating the availability of lysine from the lysine content of the feces, the lysine arising from digestive secretions was taken to be proportional to the food consumption. That this assumption is justified has been shown by Kuiken and Lyman ('48). It was also assumed that the lysine in the basal proteins was unavailable to the same extent from both the supplemented and unsupplemented diets, and, hence, was proportional to food consumption. This assumption could not be verified and it is possible that the negative values for unavailable lysine in two standard groups and the somewhat lower values obtained by this method for the two groups fed beef as a supplement (table 5) indicate that the amount of lysine arising from the "basal proteins" decreased when the supplements were added. In spite of this limitation, the re-

sults check reasonably well with the values obtained using the growth data. Also they demonstrate the validity of the assumption that the crystalline lysine added to the basal diet was completely absorbed.

Kuiken and Lyman ('48), using a different experimental technique based on the assay of lysine in the feces, have reported 100% availability of all amino acids including lysine from roast beef. In the present study the values for availability based on simple growth data ranged from 76 to 84% with different levels of beef, values which are nearer to that of 71% obtained by Schweigert and Guthneck ('53) who used protein-depleted rats. However, when weight gain per unit of food consumed was used as the basis for calculation, values of from 85 to 98% were obtained for the availability of lysine from roast beef.

The results of the determination of unavailable lysine in the feces of experimental groups 1 and 3, table 4, as shown in table 5 point to the validity of the higher availability values obtained by the latter method of computation. Further, duplicate determinations of availability from beef (table 3), and from the cereal proteins (table 4), show greater uniformity when the availability is based on weight gain per unit of food consumed. It should also be pointed out that the weights of the individual animals in each group were roughly proportional to their food intakes, and that a linear growth response was obtained by plotting weight gain against total amino acid intake irrespective of the nature of the dietary carbohydrate (fig. 1), indicating further the necessity of taking food consumption into account in determining "true" biological availability.

The values for the availability of lysine from the two different milk powders are in agreement with the observations of Mauron et al. ('55) who reported that lysine is inactivated to a considerable extent in roller-dried preparations.

The differences among the cereals merit further investigation, particularly the observation that the lysine in corn was only about 50% available. This effect cannot be attributed to

the poor digestibility of the zein fraction because zein contains no lysine. It is noteworthy, however, that the value for rice, which contains the smallest amount of alcohol-soluble proteins, was highest. The effect of adding a high percentage of starch to the diet, as was necessary in the determination on the cereal proteins, should have been negligible since autoclaved starch ("dextrin") was used as the dietary carbohydrate throughout.

SUMMARY

Values for the biological availability of the lysine in 7 different purified proteins, and the proteins of three cereals and two milk powders, were determined by measuring their value as supplements to a wheat gluten diet low in lysine.

The availability of the lysine in beef was 76%, from gelatin 74%, and that in the other purified proteins tested was 90 to 104% as calculated from simple growth data. Higher values, particularly for beef and gelatin, were obtained when availability was calculated from weight gain per unit of food consumed. Evidence was obtained suggesting that the second method of computing availability was more accurate.

Values for the availability of the lysine in three cereal proteins differed considerably, the value for rice being highest with those for wheat and corn following in order. The lysine of roller-dried skim milk was less available than that of a spray-dried preparation.

Lysine availability was not appreciably affected by changes in the level of the test protein.

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STUDIES ON ARGININE DEFICIENCY IN CHICKS

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A number of reports have been published recently suggesting that the arginine requirement of the chick is different on a practical type diet than on a purified diet. Snyder et al. ('56), reported that 1.7% total arginine was necessary in a purified casein diet for maximum chick growth, while arginine supplementation of a practical diet, calculated to contain 1.1% arginine, failed to improve growth. Krautmann et al. ('56), also reported that the arginine requirement was higher on a purified casein diet than on a practical diet or a casein-gelatin diet, and concluded that some unknown factor is present in gelatin that enhances the utilization of arginine or renders the arginine of casein more available. These observed differences in requirement may be explained by the occurrence of arginine-sparing compounds in the natural ingredients.

Almquist et al. ('41), presented evidence showing that creatine could spare arginine and glycine under their conditions, and that glycocyamine and creatinine had a sparing action to a lesser extent. This sparing action of creatine has recently been confirmed by Wietlake et al. ('54), and Fisher et al. ('56). The studies reported in this paper were carried out to determine more precisely the magnitude of the glycocyamine-sparing action and to study some other dietary ingredients that may affect its ability to spare arginine.

EXPERIMENTAL

Single-comb White Leghorn cockerels were used in these experiments. The day-old chicks were individually banded and weighed at weekly intervals during the course of the three-week experimental period. They were housed in electrically heated battery brooders with wire mesh floors. Feed and water were supplied ad libitum, and feed consumption was recorded. The composition of the basal diet used in both experiments is given in table 1.

The supplements were added to the basal diet at the expense of dextrose. The relationships of the various levels used are shown on a molecular equivalent basis.

ARGININE EQUIVALENT	ARGININE HCl	GLYCOCYAMINE	CREATINE (ANHYDROUS)	BETAINE MONOHYDRATE	DL-METHIONINE	CHOLINE Cl
0.06		0.040		0.047	0.051	0.048
0.20	0.242					
0.30		0.202		0.233	0.257	0.240
0.40	0.483					
0.60	0.726	0.403	0.452	0.465	0.513	0.481
0.80	0.968					
1.00	1.209					
1.20	1.451					

In the first experiment groups of chicks were fed glycocyamine at various levels of dietary arginine to determine if glycocyamine would substitute for a part of the chick's requirement for arginine. Any sparing action that glycocyamine might show could conceivably be attributed to creatine formation, thereby sparing arginine for protein anabolism. Therefore, experimental groups were introduced to determine if additional methyl groups, from methyl donor compounds, might be needed for the methylation of glycocyamine. Two lots of chicks were also fed creatine. In the second experiment, the supplemental value of various levels of betaine, methionine, glycocyamine and choline were compared in a casein diet low in arginine. In addition, the supplemental value of betaine was tested at various levels of dietary arginine.

TABLE 1

Composition of basal diet

INGREDIENT	AMOUNT/ 100 GM	INGREDIENT	AMOUNT/ 100 GM
	gm		*mg*
Dextrose	64.65	$MnSO_4 \cdot H_2O$	26.4
Casein	22.00	$FeSO_4 \cdot 7H_2O$	11.0
Glycine	0.80	$CuSO_4 \cdot 5H_2O$	1.1
DL-Methionine	0.30	$CoCl_2 \cdot 6H_2O$	1.1
Cellulose [1]	3.00	$ZnCl_2$	1.1
Hydrogenated fat	3.00	KI	1.1
Vitamin A concentrate		$Na_2MoO_4 \cdot H_2O$	0.11
(5,000 USP units/gm)	0.20	Inositol	110.0
Dicalcium phosphate	3.14	Para-aminobenzoic acid	11.0
Calcium carbonate	0.85	Calcium pantothenate	2.2
Iodized salt	0.75	Niacin	2.64
KCl	0.60	Thiamine·HCl	1.32
$MgSO_4$	0.255	Riboflavin	1.32
d-α-Tocopheryl acetate concentrate N.F. (250 mg/gm)	0.07	Pyridoxine·HCl	0.66
		Folic acid	0.44
Choline chloride	0.20	Menadione	0.22
		Biotin	0.044
		Vitamin B_{12}	0.022
		Vitamin D_3 concentrate (15,000 ICU/gm)	13.332

[1] Solka-Floc, The Brown Company, Berlin, N. H.

RESULTS

The results of the first experiment are shown in table 2. In all cases where the level of arginine was suboptimal for growth, a response was obtained from supplemental glycocyamine. When creatine alone was added to the basal diet a significant increase in the rate of growth of the chicks was observed. However, when creatine was added in the presence of a low level of supplemental arginine, no further improvement in growth rate was observed. In every case where betaine was added to the basal diet there was an improvement in growth, particularly when betaine was added to the diet containing 0.2% of supplemental arginine. On the other hand, methionine failed to improve the growth of chicks and in two of the three cases actually depressed growth (table 2).

TABLE 2

Arginine, glycocyamine, betaine, methionine and creatine as supplements to the casein basal diet

TREATMENT	AV. WT. 3 WKS.[1]	FEED/ GAIN
	gm	
None	68	3.50
0.2% arginine	101	2.75
0.4% arginine	120	2.59
0.6% arginine	152	2.14
0.8% arginine	156	2.21
1.0% arginine	149	2.17
0.4% glycocyamine	83	2.94
0.2% arginine + 0.4% glycocyamine	120	2.27
0.4% arginine + 0.4% glycocyamine	143	2.22
0.6% arginine + 0.4% glycocyamine	156	2.06
0.8% arginine + 0.4% glycocyamine	153	2.12
1.0% arginine + 0.4% glycocyamine	158	1.97
0.2% arginine + 0.4% glycocyamine + 0.47% betaine	132	2.31
0.2% arginine + 0.4% glycocyamine + 0.51% methionine	115	2.39
0.2% arginine + 0.47% betaine	130	2.45
0.2% arginine + 0.51% methionine	104	2.78
0.4% glycocyamine + 0.47% betaine	90	2.90
0.4% glycocyamine + 0.51% methionine	76	3.54
0.45% creatine	98	2.74
0.2% arginine + 0.45% creatine	101	2.81

[1] Fifteen hybrid cross SCWL chicks per treatment.

The experimental design and results of the second experiment are shown in table 3. A growth response was obtained from betaine at all levels of dietary arginine. This response was greatest at the lower levels of arginine and indicates that betaine reduces the chick's requirement for arginine. The results of this experiment show that 0.465% betaine promotes a greater growth response than the 0.233% level, while 0.047% had no effect. Methionine supplementation produced a growth response only at the 0.257% level indicating that in the first experiment the 0.513% of methionine was probably too high. A growth response from glycocyamine was obtained at the two highest levels of supplementation while choline was only effective at the highest level (0.481%). The growth response from betaine and methionine was significantly greater than that obtained from choline or glycocyamine.

TABLE 3

Studies on the interrelationship of betaine, glycocyamine and arginine in the chick

TREATMENT	AV. WT. 3 WKS.[1]	FEED/ GAIN
	gm	
None	83	3.06
0.2% arginine	126	2.69
0.4% arginine	165	2.12
0.8% arginine	193	1.90
1.2% arginine	201	1.88
0.2% arginine + 0.047% betaine	122	2.65
0.2% arginine + 0.23% betaine	136	2.42
0.2% arginine + 0.47% betaine	151	2.52
0.4% arginine + 0.47% betaine	184	1.92
0.8% arginine + 0.47% betaine	206	1.84
1.2% arginine + 0.47% betaine	208	1.80
0.2% arginine + 0.04% glycocyamine	130	2.44
0.2% arginine + 0.20% glycocyamine	144	2.30
0.2% arginine + 0.40% glycocyamine	142	2.42
0.2% arginine + 0.051% methionine	130	2.65
0.2% arginine + 0.26% methionine	151	2.21
0.2% arginine + 0.51% methionine	131	2.58
0.2% arginine + 0.048% choline	129	2.63
0.2% arginine + 0.24% choline	124	2.69
0.2% arginine + 0.48% choline	140	2.38

[1] Twenty inbred foundation strain SCWL chicks per treatment.

DISCUSSION

The response from glycocyamine and from glycocyamine plus betaine when fed in a diet low in arginine suggests that these compounds were being used for creatine formation in place of arginine. However, the response from betaine in the absence of glycocyamine and the lack of response from methionine in the presence of glycocyamine indicates that betaine has some action other than that of supplying methyl groups for creatine formation. If the response from betaine was due to transmethylation, then the results of these experiments suggest that the choline oxidase activity of the chick is not efficient unless large amounts of choline (0.48%) are supplied in the diet.

The difference in growth at various arginine levels obtained between experiments 1 and 2 was probably due to differences

in strain of chick. Although the chicks were obtained from the same hatchery, it was later discovered that the chicks used in experiment 1 were a hybrid cross, while those used in experiment 2 were an inbred foundation strain. Since the hybrid chicks ordinarily grow faster than those of the foundation strain, the reverse situation found in this study emphasizes the role that genetics plays in studies on intermediary metabolism using conditions of "stress" -nutrition and growth as criteria.

In view of the differences in arginine requirement observed in these experiments and experiments from other laboratories (Almquist and Merritt, '50; Young et al., '53; Wietlake et al., '54; Snyder et al., '56; and Fisher et al., '56), it would appear that the arginine requirement may be a function of rate of growth and strain and breed of chicks used.

The data from various experiments have been summarized in table 4. In order to eliminate differences in strain and breed of chicks and in rate of growth and feed efficiency, these data have been expressed as milligrams of arginine intake per gram of gain. An examination of the first 5 experiments in this table (lines 1 to 5) shows that when the diet contains suboptimal amounts of arginine, the quantity of arginine required for a gram of gain is below 32 mg. When the chick consumed approximately 32 mg of arginine per gram of gain, the diet appears adequate in arginine.

The data from experiment 1 are summarized in line 4. Poor growth and feed efficiency were obtained in this experiment, and the arginine requirement appears to be 1.4%. In the second experiment (line 5) better growth and feed efficiency were obtained, and the chicks appeared to have a higher arginine requirement of 1.6%. However, in both cases, maximum gain was obtained when the chicks obtained approximately 30 mg of arginine per gram of gain.

When very large amounts of materials such as creatine, glycocyamine, or betaine are added to the diet (lines 6, 7 and 8), it appears that the amount of arginine required per gram of gain need not be as great. With creatine in the diet, approximately 24 mg of arginine per gram of gain appears suffi-

Line No.	
1	Snyder et al. ('56) Arginine requirement 1.73%, Casein diet
2	Snyder et al. ('56) Arginine requirement 1.68%, Casein-gelatin diet
3	Fisher et al. ('56) Arginine requirement 1.9%, Casein diet
4	Experiment 1—this paper Arginine requirement 1.4%, Casein diet
5	Experiment 2—this paper Arginine requirement 1.6%, Casein diet
6	Fisher et al. ('56) Arginine requirement 1.5%, Casein diet + 1% creatine
7	Experiment 1—this paper Arginine requirement 1.3%, Casein diet + 0.4% glycocyamine
8	Experiment 2—this paper Arginine requirement 1.4%, Casein diet + 0.47% betaine

TABLE 4

various diets on the amount of arginine required to produce a gram of gain

% arginine in diet	1.31	1.40	1.48	1.56	1.64	1.73	1.81
mg arginine/gm gain	29.0	28.1	28.5	30.4	30.8	32.3	33.7
% arginine in diet	1.39	1.49	1.58	1.68	1.78	1.87	1.97
mg arginine/gm gain	30.0	27.8	30.2	29.8	33.2	34.9	38.0
% arginine in diet	0.8	1.1	1.3	1.5	1.7	1.9	
mg arginine/gm gain	20.7	21.2	22.6	25.3	27.6	28.9	
% arginine in diet	0.8	1.0	1.2	1.4	1.6	1.8	
mg arginine/gm gain	28.0	27.5	31.1	30.0	35.4	39.1	
% arginine in diet	0.8	1.0	1.2	1.6	2.0		
mg arginine/gm gain	24.5	26.9	25.4	30.4	37.6		
% arginine in diet	0.8	1.1	1.3	1.5	1.7	1.9	
mg arginine/gm gain	16.4	18.2	21.2	23.5	26.0	29.5	
% arginine in diet	0.8	1.0	1.2	1.4	1.6	1.8	
mg arginine/gm gain	23.5	22.7	26.6	28.8	33.9	35.5	
% arginine in diet	1.0	1.2	1.6	2.0			
mg arginine/gm gain	25.2	23.0	29.4	36.0			

cient, while glycocyamine (0.403%) reduces the amount to approximately 28 mg. The presence of betaine (0.465%) reduces the amount required to not more than 29 mg.

Snyder et al. ('56), reported that while the arginine requirement for chicks receiving a casein diet was 1.73%, a practical diet calculated to contain 1.11% of arginine could not be improved by arginine supplementation. From these workers' figures this would mean that with the practical diet adequate in arginine, only 21 mg of arginine produced a gram of gain. However, if either values published in National Research Council Publication No. 449 or compiled by Almquist ('57), are used for calculating the arginine content of the diet, it is found to contain approximately 1.4% of arginine. If this value is used, this diet would require 27 mg of arginine to produce one gram of gain (a value which compares favorably with the data obtained using the purified diet which contained compounds which spare arginine). It is likely that the practical diet used by Snyder et al. ('56), contains some of these arginine-sparing compounds. It is interesting to note that Snyder and co-workers added 0.20% of choline and 0.10% of DL-methionine to this diet which already contains by calculation 0.15% of choline and 0.36% of methionine. In view of the fact that these compounds give a growth response on an arginine-deficient diet, it is probable that they also will have a sparing action on the arginine requirement in a practical diet. In addition, there may well be other compounds that have a sparing action on arginine that are at present unknown.

SUMMARY

Two experiments were conducted with chicks fed a purified casein diet low in arginine, to determine the growth-promoting effect of various levels of arginine, glycocyamine, betaine, choline and methionine and certain combinations of these compounds.

A growth response was obtained from betaine at all levels of dietary arginine. This response was greatest at the lower levels of arginine. Glycocyamine produced a response only at

the lower levels of arginine and a further growth response was obtained with the addition of betaine.

Creatine gave a growth response only in the absence of supplementary arginine.

A response was obtained from the highest level of choline tested and from an intermediate level of methionine.

The differences in arginine requirement observed in these experiments and experiments from other laboratories are compared on the basis of arginine required to produce a gram of gain. The significance of this type of comparison is discussed.

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ORGAN, URINE AND FECES VITAMIN B_{12} CONTENT OF NORMAL AND STARVED RABBITS [1]

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Numerous studies have shown that rabbit blood and plasma contain considerably more vitamin B_{12} activity than the blood and plasma of other laboratory and domestic mammals so far reported (Couch et al., '50; Ross, '50; Rosenthal and Brown, '54). The vitamin B_{12} activity of rabbit liver, kidney and spleen, however, has been reported as comparable to those of the chick (Yacowitz et al., '52; Kulwich et al., '53) but the similarity is not substantiated in more recent reports (Shenoy and Ramasarma, '54). Liver and kidney tissue from rabbits, cattle and sheep also appear to contain similar concentrations of the vitamin (Moinuddin and Bentley, '55). On the other hand, beef liver may contain 10 to 20 times more vitamin B_{12} activity than rat liver (Lewis et al., '49). Differences of vitamin B_{12} activity in the organs from different animals may be attributed to species differences (Miller et al., '56) but a comparison of reports for tissues of the same species indicates a wide variation which has not been adequately studied. For example, Pitney et al. ('55) report 0.28 μg vitamin B_{12}/gm for human liver while Swenseid et al. ('57) report an average of 0.70 μg/gm of tissue. Other investigators have shown that the concentration of vitamin B_{12} in rat tissues is, in part, a function of the dietary intake (Sheid et al., '51). Little information is available concerning the physiological state of the animal and its effect on tissue vitamin concentra-

[1] Some of these data were presented before the Division of Biological Chemistry, American Chemical Society, Miami, Florida, April, 1957.

tion with the exception of thyroid feeding and vitamin depletion (Frost et al., '49; Lewis et al., '49).

In the present study, the vitamin B_{12} activity of organs, feces and urine of rabbits subjected to starvation, partial food restriction and antibiotic feeding were determined. The data which we have obtained form the basis for this report.

MATERIALS AND METHODS

New Zealand white virgin female rabbits weighing 2 to 4 kg were housed in metabolism cages for the collection of urine and feces. Urine was collected in acid to acidify the alkaline urine normally produced by rabbits. Three-day collections of urine and feces were pooled for analysis. The rabbits were sacrificed by air embolism; the tissues were weighed and frozen at 20°C until they could be prepared for analysis. Plasma samples, from blood obtained by cardiac puncture, were frozen until analyzed. Tissue and feces samples were homogenized in a blendor with 5 volumes of acetic acid buffer (0.2%, pH 4.6) and the homogenates were steamed in an autoclave for 30 minutes at 100 to 105°C. Portions of the extracts were clarified by centrifugation, adjusted to pH 6.8 $\pm$ 0.1 and frozen until analyzed. Aliquot portions of the feces homogenates were dried to constant weight at 100°C for estimation of fecal dry weight.

Vitamin B_{12} analyses were made on the plasma and urine samples, and on the tissue and feces extracts with *Lactobacillus leichmannii,* by methods previously described (Rosenthal and Sarett, '52). Estimation of deoxyriboside activity of urine and extracts were performed following destruction of the vitamin B_{12} with alkali (Hoffman et al., '48). Urinary creatinine values were determined with alkaline picrate reagents. The rabbits were fed a commercial rabbit chow,[2] containing less than 5 mμg/gm as analyzed with *L. leichmannii.* Water was available to the animals at all times and no attempt was made to prevent coprophagy.

[2] Wayne Rabbit Ration, Allied Mills Inc., Chicago, Illinois.

Neomycin hydrochloride was dissolved in the drinking water and given to the animals three times weekly. Feces bacterial counts were made by conventional bacteriological plate counting techniques using tryptose agar media for estimation of total aerobic organisms.

RESULTS AND DISCUSSION

In the first series of experiments, 8 rabbits were fed commercial rabbit chow [3] for 4 days. Urine collections were made during the last three days in order to obtain baseline urinary excretion values for vitamin B_{12}. The rabbits were then subjected to complete starvation for 13 days and urine was

TABLE 1

Effect of starvation on urinary excretion of vitamin B_{12} by rabbits

TREATMENT	BODY WT.	URINE CREATININE	URINE VITAMIN B_{12} TOTAL	ACTIVITY ALKALINE-STABLE
	kg	*mg/day*	*μg/day*	*mμg/day*
Fed	2.81 ± 0.12 [1]	153 ± 8 [1]	1.05 ± 0.12 [1]	42 ± 4 [1]
Starved	2.12 ± 0.14	122 ± 12	2.48 ± 0.26	33 ± 4
Refed	2.71 ± 0.18	114 ± 10	1.63 ± 0.34	48 ± 5

[1] Standard error of the mean. The average values obtained from 7 or 8 animals.

collected during the last three days. Following the starvation regime, the animals were fed ad libitum for an additional 13 days, urine collections being made during the last three days.

During the starvation period, the rabbits lost 25% of their body weight (table 1). The excretion of vitamin B_{12} in urine more than doubled following starvation but the excretion of alkaline-stable material decreased significantly. On refeeding, the body weight was regained and the excretion of the vitamin and alkaline-stable material returned almost to pre-starvation levels. It is interesting to note that rabbits maintained on a diet practically devoid of vitamin B_{12} excrete 375 mμg/kg of body weight/day in urine as compared with 25 mμg/kg/day for dogs (Rosenthal and Hampton, '55) and 1 mμg/kg/day

[3] See footnote 2.

for man (Register and Sarett, '52; Unglaub et al., '54). Although the creatinine excretion decreased to some extent during the experiment, the decrease is of doubtful significance and the evidence is good that urine collections were essentially complete. It was observed that urine volume and the amount

TABLE 2

Effect of starvation on tissue vitamin B_{12} in rabbits

	NORMAL (8)	STARVED (8)	REFED (8)	NEOMYCIN [1] (4)
Body weight (kg)	2.61 ± 0.06 [2]	1.91 ± 0.18 [2]	2.72 ± 0.18 [2]	3.05 ± 0.25 [2]
Kidneys (2)				
gm/100 gm B. W.	0.635	0.613	0.627	0.674
Total B_{12}, mμg/gm	383 ± 7.9	2714 ± 421	944 ± 178	431 ± 94
Alkaline-stable, mμg/gm	9 ± 1	16 ± 2	149 ± 31	6 ± 0.5
Liver				
gm/100 gm B. W.	3.63	2.32	3.34	3.38
Total B_{12}, mμg/gm	371 ± 37	785 ± 70	610 ± 68	323 ± 27
Alkaline-stable, mμg/gm	18 ± 6	21 ± 8	105 ± 24	19 ± 3
Heart				
gm/100 gm B. W.	0.206	0.226	....	0.181
Total B_{12}, mμg/gm	165 ± 27	331 ± 33	....	281 ± 78
Alkaline-stable, mμg/gm	6 ± 0.3	6 ± 1.2	....	3 ± 0.6
Spleen				
mg/100 gm B. W.	0.750	0.256	....	0.700
Total B_{12}, mμg/gm	180 ± 17	165 ± 15	....	56 ± 9
Alkaline-stable, mμg/gm	22 ± 4	16 ± 2	....	13 ± 2
Blood plasma,[3] mμg/ml	17 ± 3	25 ± 3	....	20 ± 2

[1] After 42 days of neomycin feeding.
[2] Standard error of the mean.
[3] Five animals.

of water consumed increased considerably during the starvation period but urine volume measurements were not made. Also during starvation, some of the animals became edematous.

In a second study, untreated control rabbits were sacrificed in order to obtain baseline vitamin B_{12} values of some of the organs (table 2). Other animals were starved for 10 days,

and some of the starved rabbits were fed stock diets ad libitum for an additional 10 days before obtaining tissue for analysis. The most striking observation concerns the kidney vitamin B_{12} content which increased from 383 mμg/gm to 2714 mμg/gm following starvation. On realimentation, the kidney vitamin content approached normal values but was still significantly elevated after 10 days of refeeding. Although the content of alkaline-stable material represented less than 3% of the total activity, in all of the tissues studied, a significant increase in this material was observed in the refed animals. Apparently, refeeding is associated with regeneration of cellular material with concomitant increases in the concentration of nucleic

TABLE 3

Effect of food intake on feces vitamin B_{12} in rabbits

INTAKE	WT. FOOD EATEN	WT. DRY FECES	TOTAL VITAMIN B_{12} IN DRY FECES	ALKALINE-STABLE VITAMIN B_{12} IN DRY FECES
%	*gm*	*gm/day*	*μg/gm*	*μg/gm*
100	119 ± 6 [1]	46 ± 3 [1]	2.6 ± 0.3 [1]	0.14 ± 0.04 [1]
75	78 ± 10	23 ± 3	2.7 ± 0.6	0.10 ± 0.05
50	63 ± 2	19 ± 5	3.1 ± 0.6	0.04 ± 0.01
25	29 ± 2	8 ± 2	3.2 ± 0.7	0.16 ± 0.05

[1] Standard error of mean. The average values derived from 4 to 7 determinations.

acids. The vitamin B_{12} content of liver and heart tissue also increased above basal values but to a lesser extent than kidney tissue. The vitamin content of the spleen remained constant although the organ became considerably smaller.

The relationship between the amount of food consumed and feces vitamin B_{12} content was studied by feeding the animals at libitum for 5 days, collecting feces during the last three days. After establishing the amount of food consumed, the animals were offered graded amounts of food for 5-day periods with collections during the last three days. In order to avoid undue loss of weight, high and low amounts of food were offered during alternate periods. It is apparent (table 3) that the amount of feces produced is related to the amount of food consumed. The daily feces vitamin B_{12} excretion decreased

from 119 μg when fed ad libitum to 26 μg when fed 25% of the ad libitum intake. However, the concentration of vitamin B_{12} in feces remained essentially constant at all dietary intakes averaging 2.9 μg per gram of dry feces. The concentration of alkaline-stable material present in rabbit feces, representing less than 8% of the total vitamin activity, was not altered by restriction of dietary intake. Whether the alkali-labile microbiologically-active material in rabbit feces is cyanocobalamin or closely related substances must await further study.

Numerous studies indicate the sparing effect of antibiotics on vitamin B_{12} requirement and storage in various animals. These effects depend not only on the antibiotic used but also on the species of animal, and in many instances have led to equivocal information (Smith, '54). It was of interest to us, therefore, to study blood, organ and feces vitamin B_{12} content of rabbits fed neomycin base, for as long as 7 weeks.

On feeding neomycin, the bacterial flora of the feces decreased from 10^9 organisms/gm fresh feces to 10^5 organisms for the duration of the experiment (fig. 1). The bacterial count returned to normal numbers within 5 weeks after the antibiotic was discontinued. During the time the neomycin was fed, the amount of feces produced decreased somewhat due to lower food consumption. The concentration of total vitamin B_{12} activity and alkaline-stable activity, however, remained essentially constant within experimental error. When the antibiotic was discontinued (after 5 weeks) food consumption increased to control values but the concentration of total and alkaline-stable vitamin B_{12} activity decreased markedly. It is conceivable that the rapid increase in numbers of vitamin B_{12}-requiring organisms exceeded the production of the vitamin in the gastrointestinal tract although other explanations are tenable.

After 6 weeks of neomycin feeding, 4 rabbits were sacrificed and tissue vitamin B_{12} analyses were made (table 2). The concentration of vitamin B_{12} activity in plasma, liver and kidney tissue were the same as in the control animals. Heart tissue, however, contained slightly higher amounts

($P = < 0.05$) and spleen considerably less activity ($P = < 0.01$) than control animals. For animals fed neomycin, the tissues remained the same size in relation to body weight as in the normal controls.

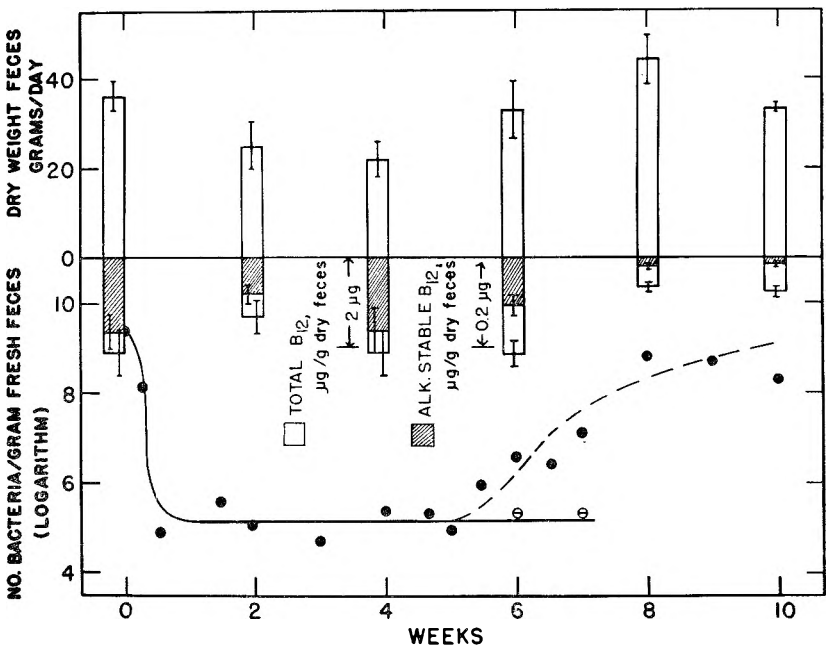

Fig. 1 Feces dry weight, vitamin concentration and bacterial count before, during and after neomycin feeding. Neomycin was discontinued at 5 weeks for 4 animals (●) but continued to 7 weeks for 4 other animals (⊖). Values for first 5 weeks obtained with 8 animals and for last 5 weeks on 4 animals. The vertical lines represent one standard error.

It is well known that vitamin B_{12} is tenaciously held for long periods of time by the organs of man and laboratory animals thus indicating a slow rate of turnover. The increased concentration of vitamin activity in the kidney, liver and heart may indicate an increased requirement for vitamin B_{12} during starvation. It is possible that each organ retained the vitamin while the organ mass was decreasing with an apparent increase in concentration per gram of tissue. Calculation of the data from table 2 show that the kidney, liver,

heart, and spleen lost 30, 54, 21, and 73% of their weight during starvation. The total kidney vitamin B_{12} activity during starvation increased from 0.6 to 32 μg, a 50-fold increase while the total heart vitamin only increased from 0.7 to 1.4 μg. On the other hand, the vitamin B_{12} content of the total liver (35 μg) remained constant while that of the spleen decreased from 0.33 to 0.08 μg. These data strongly indicate that the vitamin B_{12} content of the organs is variable and related to the physiological state of the animal.

The large urinary excretion of vitamin B_{12} by rabbits consuming a diet practically devoid of the vitamin indicates bacterial synthesis in the gut. It has previously been reported by Kulwich et al. ('53) that rabbits excrete between 50 and 100 μg of vitamin B_{12} daily in feces and that the soft or night feces (which are eaten) contain two to three times as much of the vitamin as hard feces. Presumably, vitamin B_{12} is synthesized in the gastrointestinal tract by microorganisms. In the experiments reported here, the animals were permitted to consume the night feces, thus obtaining considerable quantities of vitamin B_{12}. Even during starvation, small amounts of feces, presumably night feces, are produced and eaten immediately and are never observed in the metabolism cages unless the animals are collared.

It is apparent from these experiments that starvation has a marked effect on the distribution of vitamin B_{12} activity in the organs of rabbits, but no effect on plasma vitamin B_{12}. These effects appear to be largely reversible. On the other hand, feeding neomycin for 6 weeks decreased spleen vitamin activity, but has no effect on the distribution of the vitamin in either kidney or liver tissue. During the starvation period, intestinal synthesis of vitamin B_{12} can only be assumed to have been minimal. Since vitamin B_{12} synthesis by animal tissues has not been demonstrated, we can only assume that the increased activity of the tissues was derived from labile stores which released the vitamin during starvation.

SUMMARY

Starvation of rabbits resulted in increased concentration of vitamin B_{12} activity in kidney, liver, heart, and urine but plasma and spleen vitamin activities are not altered from normal. Refeeding tends to return values to normal. The vitamin B_{12} activity of kidney, liver, heart or feces is not affected by feeding neomycin to suppress bacterial growth in the gut, but spleen vitamin activity is reduced. The concentration of vitamin B_{12} in feces is not affected by the amount of food consumed or feces produced.

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DEPENDENCE OF BIOLOGICAL VALUE ON PROTEIN CONCENTRATION IN THE DIET OF THE GROWING RAT

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The biological value of protein may be measured in a variety of ways (Mitchell, '52; Allison, '55; Miller and Bender, '55) and under rigidly standardized conditions can be depended upon to reflect accurately the rank of growth-promoting capacity of the protein sources for the species and age of animal under investigation. In few cases, however, are such determinations made under conditions optimum for growth of the experimental subject, the major limitations ordinarily being those of a restricted food intake and sub-optimum protein intake. While these restrictions have been useful by providing standardized conditions for purposes of comparison and in promoting sensitivity of the test, they have at the same time limited the scope of applicability of the experimental data. Osborne, Mendel and Ferry ('19) concisely expressed the view that, "Economy of food can be effected only by supplying the young animal with as much as it will eat; economy of protein only by reducing the nutritive ratio below that at which the normal growth can be maintained." If the biological value is to have more than pedagogical significance we must have some means of translating the data obtained under specified, artificial conditions to practical situations.

The effect of energy intake on biological value seems important only at the extreme situation in which protein must be called upon to provide energy for vital functions (Mitchell, '43; Forbes and Yohe, '55). Upon this view there seems to be

general agreement. The effect of different protein concentrations in the diet on the biological value has also been studied (Mitchell, '24; Barnes and Bosshardt, '46), but the quantitative aspects of this concept have not been thoroughly explored. It was with these viewpoints in mind that the following studies were initiated.

EXPERIMENTAL METHODS

The experiments reported here involved the determination of the biological value [1] of three different proteins fed in varying concentration to growing albino rats.

The proteins employed in these experiments were: (a) whole egg, prepared in our laboratory by exhaustive hexane extraction of whole, steam-cooked, dried egg (shells not included), (b) vitamin-free casein [2] and (c) hexane extracted, ground whole peanuts.[3] The diets were, except for the proteins used, identical with those described by Forbes ('54). They were designed to contain 4% mineral, 2% fiber and 10% ether extract. Protein was incorporated into the diets at the expense of equal portions of the two carbohydrates of the diet on an equal weight basis. Seven levels of peanut meal protein, 6 of egg protein and 10 of casein were studied, with either 5 or 6 animals on each level of protein except for the three lower levels of egg which were fed to 10 rats. The animals were in all cases weanling male albino rats of the Sprague-Dawley strain and were fed a stock diet for a week before being fed the experimental diets. Controlled feeding was practiced, with all animals receiving 7 or 8 gm of food daily except in a portion of the casein experiment in which two rats on each protein level were fed 6, 8, or 10 gm of food daily. In these experiments the rats were first fed for 14 days on the low-

[1] The term "biological value" as used in this paper may be defined as the percentage of truly absorbed nitrogen used by the body for growth and maintenance, and is calculated from the following equation: $BV = \frac{NA - WN}{NA} 100$, in which NA = truly absorbed nitrogen and WN = urinary nitrogen in excess of that excreted on a nitrogen-free diet, correction being made for change in metabolic size of the animal between the standardizing and test periods.

[2] Labco, The Borden Company.

[3] Special preparation, Viobin Corporation, Monticello, Illinois.

nitrogen standardizing diet and then for a similar period on the test diet of interest. Collection of urine and feces was made during the second week of each period.

Some of the casein-fed and peanut meal-fed animals were continued on their test diets until they had made 100 gm of body gain or for 6 weeks, at which time they were slaughtered and the carcasses analyzed for total nitrogen, ether extract and gross energy.

RESULTS

The true digestibility of the proteins was not affected by protein concentration in the diet and was about 95% for both the peanut and the egg protein and was 99% for casein, irrespective of protein level.

The biological values, and their standard errors, obtained in this series of experiments are shown in table 1, together with the linear regression equations for each protein. In making this latter calculation the two lower levels of egg protein are not included since their results obviously deviate from the linearity of the remaining egg data. Except for egg protein at the 4 and 8% levels, the biological value decreased regularly as the protein concentration increased above 4%. The regression equations (Y equals biological value, X equals per cent protein in the diet) are: $Y = 126.5 - 2.73X$; $Y = 96.1 - 1.78X$; and $Y = 75.3 - 1.35X$ for egg, casein and peanut protein, respectively. These data are presented graphically in figure 1. Statistical analysis reveals the slopes of these regression lines to differ (P less than 0.01) from one another. Thus, not only does the biological value of these proteins depend on their concentration in the diet, but the quantitative relationships between them depend on the level at which they are compared. The variation in food intake imposed in the casein experiments yielded no statistically significant effect on biological value although there was a tendency for the data obtained on 6 gm of food intake to be lower than at either 8 or 10 gm at 4 of the 6 protein levels investigated.

It is of interest to compare biological values obtained under the usual, standardized conditions of 10% dietary protein with those obtained at "practical" feeding levels. It has been previously shown (Mitchell and Beadles, '52) that 10% of egg protein will support maximum growth of the albino rat.

TABLE 1

Effect of protein level in diet on its biological value for the young albino rat

PROTEIN SOURCE	AMOUNT OF PROTEIN IN DIET	NO. OF OBSERVATIONS	BIOLOGICAL VALUE	REGRESSION OF BV (Y) ON PROTEIN % (X)
	%			
Dried, extracted whole egg	4.2	10	97 ± 1.0	Y = 126.5 — 2.73X
	8.3	10	97 ± 0.4	Se = 3.16
	12.4	10	92 ± 1.1	Sb = 0.125
	16.4	5	83 ± 1.4	
	20.5	5	71 ± 1.0	
	24.5	5	59 ± 1.3	
Vitamin-free casein	4.2	5	92 ± 3.4	Y = 96.1 — 1.78X
	7.1	5	83 ± 2.3	Se = 4.93
	9.4	5	76 ± 2.2	Sb = 0.086
	11.6	6	75 ± 1.5	
	12.0	5	73 ± 1.1	
	15.1	6	69 ± 1.1	
	18.4	6	65 ± 1.1	
	21.8	6	56 ± 2.7	
	25.1	6	49 ± 2.6	
	28.9	6	47 ± 1.0	
Peanut meal	4.2	5	74 ± 1.6	Y = 75.3 — 1.35X
	6.4	5	68 ± 2.8	Se = 3.68
	8.2	5	61 ± 1.0	Sb = 0.088
	12.6	5	55 ± 0.7	
	16.4	5	52 ± 0.9	
	20.6	5	49 ± 0.7	
	24.5	5	44 ± 0.8	

In the growth studies conducted as a portion of these experiments maximum weight gain and nitrogen retention occurred at about 22% of casein and in excess of 24% of peanut meal protein (the highest level fed). The data of table 1 indicate a difference of —22% and —18%, respectively, when these proteins are fed at levels approximating the requirement for

maximum performance with the protein in question. It may be inferred from these data that as the amino acid balance of dietary protein improves, and hence a lower concentration of protein is required in the diet for maximum performance, the more nearly will the biological value determined at a conventional 10% level approach that prevailing at the practical feeding level.

Further studies are required to ascertain whether the slope of the regression of biological value on protein concentration

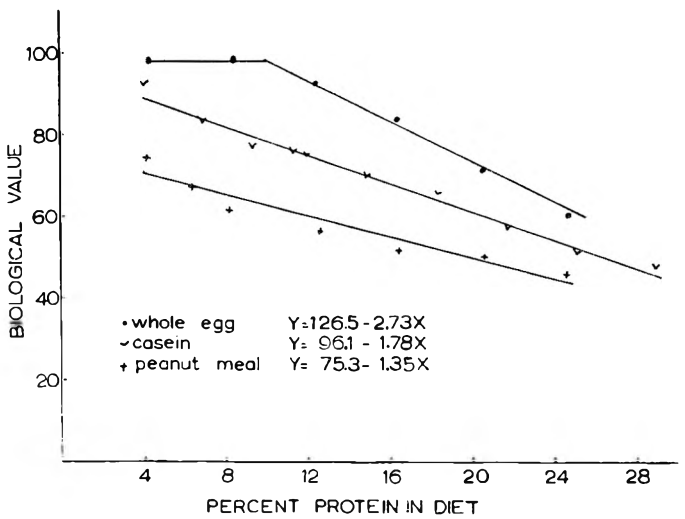

Fig. 1 Relationship between biological value and protein concentration in the diet, both expressed as percentages.

is dependent on the biological value alone. Methionine was the major limiting amino acid in the casein and peanut meal proteins used in these experiments. If it should be found that the rate of change of biological value with change in protein level is not measurably dependent on which amino acid(s) is in primary deficient supply, then the biological value of proteins or mixtures of proteins could be predicted from the biological value determined at a specified level and knowledge of the slope of the line appropriate to the determined biological value.

Another method of expressing the data of nitrogen balance experiments involves the relationship of truly absorbed nitrogen and nitrogen balance (Allison and Anderson, '45). The slope of the line expressing this relationship represents the nitrogen balance index and is to all intents and purposes synonymous with biological value under appropriate circumstances, the most important being that the data be linear, including data (NE_0) obtained on the low-nitrogen diet used for establishing endogenous and metabolic nitrogen data for

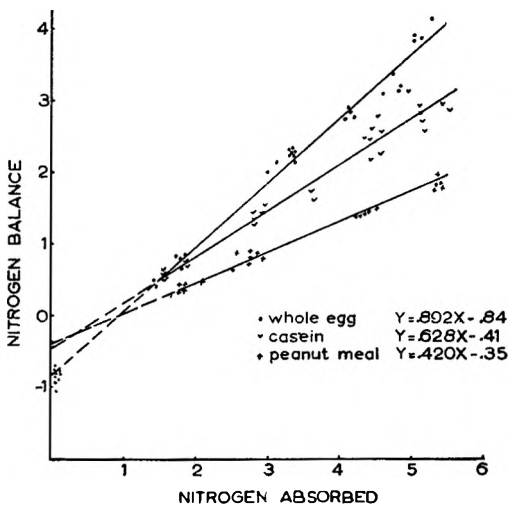

Fig. 2 Linear expression of relationship between nitrogen balance and absorbed nitrogen. Partial data, expressed as milligrams of nitrogen per gram body weight raised to the ¾ power.

the biological value calculation. Figure 2 and table 2 show the results of this method of treating the data of the present experiment.

Figure 2 clearly shows the linearity of the data obtained between the limits of 1.5 to 5.5 mg nitrogen absorbed per gram body weight raised to the three-fourths power, corresponding to about 4 to 13% of protein in the diet for all three protein sources. It also clearly shows that only in the case of data derived from whole egg does the extrapolation of the experimental data to zero nitrogen intake (NE_0) yield results sim-

ilar to those actually observed on a "nitrogen-free" diet or on a 4% egg diet, which with adequate preparation of the experimental subjects leads to nitrogen excretion equal to that on a nitrogen-free diet. The same situation has been found to exist in data presented by Mitchell ('55) related to nitrogen balance of rats fed beef or egg protein. These data are presented in tabular form in table 2 in which the observed values are those actually obtained on nitrogen-free

TABLE 2

Comparison of nitrogen balance index equations calculated with (Y_1) and without (Y_2) data obtained in nitrogen-free feeding period (NE_0)

PROTEIN SOURCE AND REFERENCE	NITROGEN BALANCE INDEX EQUATION		VALUES FOR NE_0	
	With NE_0	Without NE_0	Observed	Calculated
Whole egg— this exp.	$Y_1 = 0.892X - 0.84$	$Y_2 = 0.902X - 0.88$	— 0.77	— 0.84
Casein—this exp.	$Y_1 = 0.628X - 0.41$	$Y_2 = 0.720X - 0.80$	— 0.95	— 0.41 [1]
Peanut—this exp.	$Y_1 = 0.420X - 0.35$	$Y_2 = 0.517X - 0.73$	— 0.95	— 0.35 [1]
Beef— Mitchell ('55)	$Y_1 = 0.603X - 0.29$	$Y_2 = 0.720X - 0.89$	— 0.98	— 0.29 [2]
Whole egg— Mitchell ('55)	$Y_1 = 0.893X - 1.16$	$Y_2 = 0.890X - 1.06$	— 0.98	— 1.16

[1] $P = 0.01$.

[2] $P = 0.05$

Y equals nitrogen balance. X equals nitrogen absorbed. All data expressed as milligrams per gram of body weight raised to ¾ power.

or 4% egg diets and the calculated values are obtained by extrapolation. Thus, except for egg protein, the biological values and nitrogen balance indices are not identical for the growing rat fed levels of protein of 4% or more of the diet, since the nitrogen excretion on nitrogen-free diet obtained by extrapolation differs significantly from that observed.

It may also be pointed out that the extrapolated data lead to the conclusion that nitrogen equilibrium may be obtained by the absorption of less casein or peanut meal nitrogen than of egg nitrogen, a situation at odds with the facts.

In view of the failure of extrapolation of these data in a linear fashion to yield realistic figures, one may theorize that the data selected are curvilinear rather than linear, a fact obscured by having omitted the data above 5.5 mg of nitrogen absorbed per gram body weight raised to the three-fourths power on account of their obvious departure from linearity. To test this theory, all of the data obtained in the biological value experiments were examined for curvi-

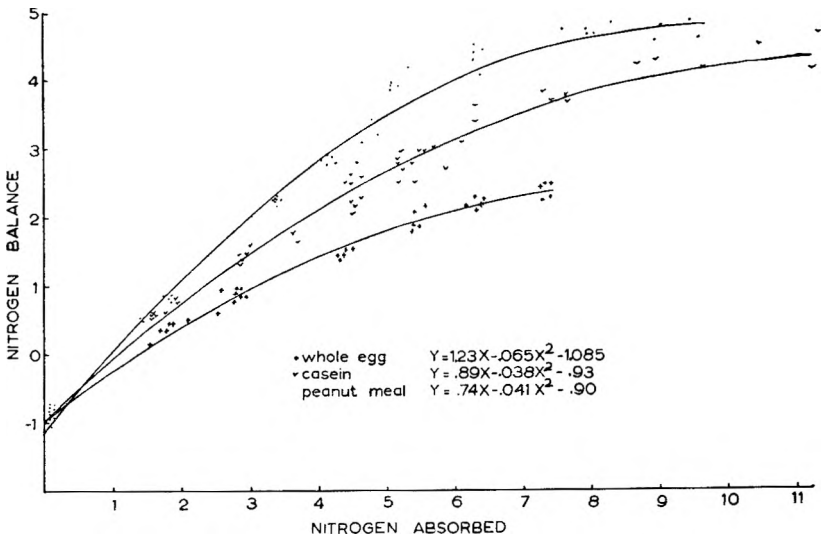

Fig. 3 Curvilinear expression of relationship between nitrogen balance and absorbed nitrogen. Total data, expressed as milligrams of nitrogen per gram body weight raised to the $\frac{3}{4}$ power.

linearity of response of nitrogen balance to nitrogen absorbed. The results of this test are shown in figure 3. In the case of each protein source the curvilinearity of regression was highly significant. When treated in this fashion the data show that as the rate of nitrogen absorbed increases the nitrogen balance increases at a decreasing rate, a finding compatible with the data for biological value. While it is true that data obtained at relatively low levels of nitrogen intake appear essentially linear they actually are sufficiently curvilinear to invalidate the concept of identity of nitrogen balance index and biological

value for growing animals except at extremely low levels of nitrogen intake. This does not invalidate the concept, however, for the adult animal in which measurements are made in the region of nitrogen equilibrium. It is only in this latter condition that the "tangent to the curve relating nitrogen intake to nitrogen balance" (Allison, '55) passes through the point of nitrogen excretion on a nitrogen-free diet.

An exception to the above statements is seen in the data obtained with whole egg protein, whose amino acid balance is eminently well suited to the needs of the growing rat. In this situation the decrease in efficiency with which the protein is used does not become apparent until after the protein requirement has been met. One may now theorize that the cause of the decreasing rate of nitrogen balance increase, hence lowering efficiency, as nitrogen absorption of incomplete proteins increases may be explained on the basis of dilution of endogenously available amino acids with successively increasing proportions of an unbalanced selection of dietary amino acids, thus in effect reducing the balance of the total mixture of amino acids available to the tissues.

Another possible explanation relates to the differential protein stores of rats fed different levels of total nitrogen. It is well-known that the level of dietary protein requirement for maintenance of nitrogen equilibrium in adult animals can be markedly affected by previous nutriture (Allison, '55), nitrogen equilibruim being obtained in protein-depleted animals on one third as much nitrogen as in normally fed animals. In the present experiment the same directional effect may be present since all animals underwent a low-protein standardizing period prior to being given the experimental diets varying in protein content. Hence, at the time of collection of excreta there was probably a graded series of labile protein stores which, as the stores increased with increasing protein intake, would tend to lower the ratio of nitrogen balance to nitrogen absorbed and thus the biological value.

It is worth while at this point to speculate on the reason for more efficient use of dietary nitrogen by protein-depleted animals. This could be explained readily if it could be shown that the indispensable body protein is characterized by a longer half-life, or slower turnover rate, than the dispensable protein stores. It is well-known that the most labile protein stores are located in intestine, liver, kidney, and muscle (Addis et al., '36; Winnick et al., '48). This has been shown by tissue analysis for total nitrogen and by isotope dilution studies. It has also been recently shown, in long-term studies employing tritium as a tracer (Thompson and Ballou, '56), that these same tissues contain the highest percentage of short-lived protein components, and the lowest percentage of long-lived components. It is then within the realm of reason to conclude that indeed the indispensable protein stores do have a lower turnover rate than do the dispensable stores and hence they may be maintained at a smaller replacement rate. Further evidence supporting this line of thought is seen in the relative inertness of nucleoproteins as shown by the increased concentration of pentose nucleic acid and deoxyribonucleic acid phosphorus in tissue protein as a result of protein deficient diets (Leslie, '55). This again supports the concept of a slower net catabolism of the more vital tissue components which would lead to a greater retention of a given quantity of absorbed protein by a growing or protein-depleted animal.

SUMMARY

The biological values and the ratio of nitrogen balance to absorbed nitrogen of whole egg, casein and peanut meal proteins were determined at concentrations between 4 and 29% of the diet, employing young albino rats as experimental subjects.

The relationship of biological value and protein concentration was linear for all data except 4 and 8% egg protein, and the equations expressing this are: $Y = 126.5 - 2.73X$; $Y = 96.1 - 1.78X$ and $Y = 75.3 - 1.35X$, in which Y is biological

value and X is percentage of protein in the diet, for egg, casein and peanut meal, respectively. The ratio of nitrogen balance to absorbed nitrogen was found to be best described by the following equations: $Y = 1.23X - .065X^2 - 1.085$ for egg, $Y = 0.89X - 0.038X^2 - 0.93$ for casein, and $Y = 74X - 0.041X^2 - 0.90$ for peanut meal proteins, in which Y is nitrogen balance and X is nitrogen absorbed, both expressed in milligrams of nitrogen per gram body weight raised to the ¾ power.

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ADAPTATION TO DIFFERENT CALCIUM INTAKES IN DOGS[1]

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Current estimates of the calcium needs of adult human beings are based entirely upon balance studies. The most recent National Research Council recommended daily allowance ('53) of 800 mg per day was chosen, on the basis of the available balance data, as the amount required to provide equilibrium in the average adult upon whom balance data were available. However, Hegsted et al. ('52) found that adult Peruvians who had lived upon low-calcium diets for long periods required only 100 to 200 mg of calcium per day to maintain balance and proposed that the calcium required for maintenance of balance is simply a reflection of the previous dietary intake and of no significance as an indication of actual need (Hegsted, '54). Many other data support the view that calcium balance in human beings is achieved at low levels of calcium intake (Basu et al., '39; Nicholls and Nimalasurina, '39; Owen et al., '40; Potgieter, '40; Walker et al., '48) and no abnormality attributable to calcium deficiency has been reported in such peoples (Walker and Arvidsson, '54; Higginson, '54). It is apparent, in retrospect, that all diets which support adult populations over long periods must provide sufficient calcium to permit balance.

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Henry and Kon ('53) demonstrated that rats which had been raised upon diets containing 0.4 to 0.8% of calcium were unable to maintain balance when placed upon a diet containing 0.13% of calcium for three months, although the extent of negative balance decreased during this period. On the other hand, rats fed the low-calcium diet throughout their lives remained in slightly positive balance. Nicolaysen ('56) also demonstrated an adaptation to low-calcium diets that persisted in old rats until death.

In the present study the ability of dogs to adapt to different levels of calcium intake, as determined by calcium balance, has been investigated. As expected, dogs fed low-calcium diets over long periods of time require exceedingly small amounts of calcium for maintenance. No pathological lesions attributable to calcium deficiency were found in such animals.

EXPERIMENTAL

A litter of 6 mongrel dogs two to three months of age was used. The animals were dewormed, dusted with an insecticide and vaccinated against distemper before being admitted to the laboratory where they were housed in individual metal cages with raised expanded metal floors. The dogs were fed a basal diet consisting of casein 20, sucrose 64.3, corn oil 9, cod liver oil 1, celluflour 3, Jones-Foster ('42) salt mix ($CaCO_3$ removed) 2.5, and choline 0.2. Vitamin supplements consisted in milligram per kilogram of ration of thiamine, 4; riboflavin, 8; pyridoxine, 4; niacin, 40; Ca pantothenate, 20; folic acid, 1, and biotin, 0.2. The dogs were divided into groups of two and appropriate amounts of $CaCO_3$ were added to the basal mix so that the groups received approximately 0.114, 0.634 and 1.234% of Ca, respectively, in their diet. The dogs were fed these diets for 34 months. At intervals during this period calcium balance studies were made using varying levels of calcium in the test diets. When not used in balance studies, the dogs were maintained on their original basal diets. During most of the balance studies, 6- to 7-day urine and fecal collec-

tions and samples of the actual diet were analyzed, but during the last year the collection periods were extended to two weeks. At the conclusion of the experiment fat-free femur, humerus and rib ash determinations and gross and histologic examinations were made of 5 of the dogs. The 6th, fed the low-calcium diet, died suddenly of undetermined causes after having been maintained in apparently excellent health for 31 months. Femur ash was obtained on this animal.

RESULTS AND DISCUSSION

No differences as a result of having been fed diets varying in calcium content from 0.114 to 1.234% could be detected in the results of fat-free bone ash determinations or in the growth rates. Femur ash values ranged from 57.3 to 58.8%, humerus 57.7 to 58.6% and rib 41.2 to 51.2%. The rib ash of the dog on the low-calcium diet was 50.3%. Weight gains during the fast growing period of the first 7 months of the experiment were 7.2 and 8.6 kg for the dogs fed 0.114% Ca, 11.2 and 7.8 kg for those fed 0.634% Ca and 6.7 and 8.2 kg for those fed 1.234% Ca. Included in the histologic examinations were inspections of the parathyroids, costo-chondial junctions, distal femoral epiphyses and metaphyses, femoral shafts and metatarsals. There were no significant differences grossly or microscopically except for mildly thickened costo-chondral junctions with slightly irregular lines of ossification suggestive of mild healed rickets in one dog fed 0.634% Ca and one fed the 1.234% Ca diet. No significant differences in weight of the parathyroids were seen. The parathyroids were composed uniformly of chief cells. The kidneys appeared normal although mild fat deposition was observed in the ascending limb of Henle's loop in the kidneys of all the animals. All other organs examined were normal.

The first balance trial was performed after the dogs had been on experiment for 4 months. The second trial was performed after 7 months. Because of the marked difference in the results obtained during the first trial while animals were

growing rapidly and afterward, the data from the first trial are presented separately (table 1) while the second and all succeeding trials are included together.

Table 1 shows that during the growth period the retention of calcium was greater in the dogs fed the high-calcium diet. However, the dogs fed the low-calcium diet were much more efficient and retained 90% of the calcium in the diet compared to only 27% in the high-calcium group. Thus, the difference in total retention between the groups is much less than the difference in the intake.

Figure 1 is a scatter diagram of the calcium intakes and excretions of the 6 dogs. As has been indicated these data were collected from the time the dogs had been fed the diets for

TABLE 1

Calcium balance data on rapidly growing dogs

CA IN DIET	CA INTAKE	CA EXCRETION	RETENTION	
%	*gm/day*	*gm/day*	*gm/day*	*% intake*
0.114	0.63	0.06	0.57	90
0.634	1.62	0.87	0.75	46
1.234	2.67	1.96	0.71	27

7 months (10 months of age) until the 34th month. No effect of age was apparent from the data during this period. In constructing these charts, logarithms times 100 of both the intake and excretion in grams per day have been used rather than the actual values, in order to provide a convenient scale for presenting all of the data and at the same time give a reasonable separation at the lower end of the scale, the region of primary interest. The regression line calculated by the method of least squares for the data from each dog is shown. The broken lines are placed one standard deviation on either side of the regression lines.

The point at which the regression line crosses the line $y = x$ is the estimated amount required to maintain balance in the animal. Since the scales are in logarithms the predicted

excretion at zero intake, as in the previous paper (Hegsted et al., '52), cannot be determined. Rather, the predicted excretions at an intake of 10 mg of calcium per day are shown (table 2) together with the predicted requirement for balance.

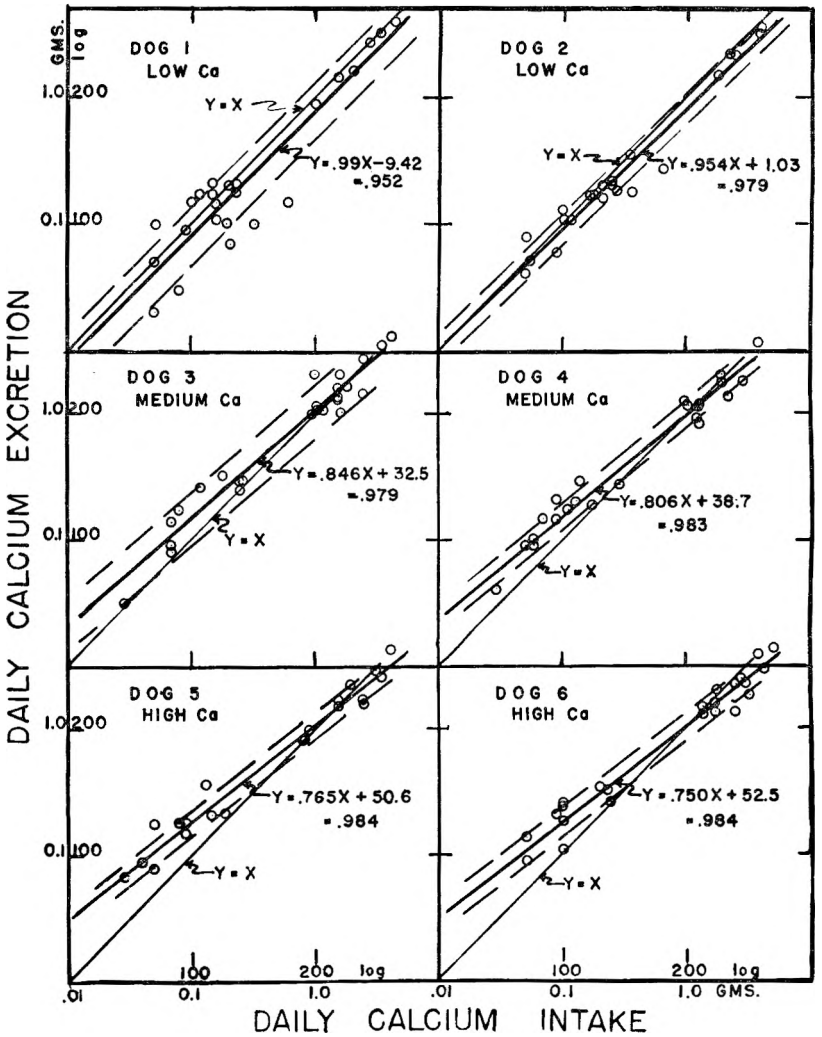

Fig. 1 Total calcium excretion at various levels of calcium intake in 6 dogs. The heavy line represents the regression line calculated by the method of least squares. One standard deviation on either side of the regression line is indicated by the broken lines.

As expected, the predicted calcium requirement for balance is to a considerable extent a reflection of the previous dietary intake. The dogs "adapted" to the low calcium-diet required very little calcium to maintain balance and a consistent negative balance was not produced by the lowest calcium diet we could conveniently prepare (0.034% Ca) which allowed intakes of the order to 50 mg per day. It will be seen (fig. 1) that the regression lines for the data on the low-calcium dogs are

TABLE 2

The effect of dietary calcium intake on the calcium metabolism of young adult dogs

DOG	CA IN DIET	FINAL WT.	DAILY CA REQUIRED FOR BALANCE		CA EXCRETION AT INTAKE OF 10MG/DAY	
	%	*kg*	*gm/dog*	*gm/kg*	*mg*	± *1 s.d.*
1	0.114	10.84	0.01	0.001	8.0	13.9 4.7
2	0.114	9.92	0.017	0.002	10.2	13.6 7.7
3	0.634	13.22	1.28	0.097	21.1	12.9 34.5
4	0.634	9.86	0.99	0.101	24.4	16.8 35.4
5	1.23	9.46	1.43	0.151	32.1	24.9 40.7
6	1.23	11.70	1.25	0.107	33.5	26.0 42.0

essentially parallel to the balance line, $y = x$. One standard deviation about the line of regression includes the line, $y = x$, over the entire range of this study. Thus, one cannot show that the regression line is different from the line of balance. This is to say that, as far as the data are concerned, the dogs were not consistently in balance or out of balance at any level of calcium intake tested. Thus, the actual values calculated as the requirements, less than 10 mg for dog 1 and 17 mg for dog 2, have little statistical validity. On the other hand, the calculated excretions at an intake of 10 mg per day are more mean-

ingful. Dog 1 would be expected to excrete from approximately 5 to 14 mg per day while dog 2 should excrete from 8 to 14 mg. Whatever the minimum excretion of these animals may be, it must be very low.

Perhaps the most unexpected finding in these animals was that they did not go into a strong positive balance at high-calcium intakes. This finding may be viewed as supporting the analytical data upon the bones; namely, that they were not depleted and had no calcium deficit to replenish.

The findings on the other animals are more straightforward. While the estimated need, however calculated, rises with the continual consumption of higher calcium levels, there is not a proportional increase. Thus, the estimated needs of dogs 5 and 6 are not greatly higher than those of dogs 3 and 4.

The results of these studies are in agreement with practically all recent calcium metabolism studies. They show that the amount of calcium required to maintain balance is largely a reflection of the customary calcium intake and that animals accustomed to small intakes have a very low and practically unmeasurable calcium need for maintenance. The numerous studies demonstrating a similar situation in man have already been referred to in the introduction. Kinsman et al. ('39) have shown that the maintenance need for children was very low. Thus, it appears that when considerable calcium is needed for new bone formation, calcium is efficiently utilized. The growing animal behaves in a manner similar to an animal adapted to low-calcium intakes.

The results may be interpreted as supporting the contention of Henry and Kon ('53) that adaptation to low-calcium diets, at least during the growth process, results in a deposition of calcium in a more stable form. The calcium content of the bones of the low-calcium animals was similar to that of those fed more liberal amounts suggesting a similar amount of total body calcium. This was demonstrated much more rigorously by Henry and Kon ('53) by total body analyses in rats. Thus, there is no direct evidence that the greater avidity with

which calcium is retained in such animals is a reflection of smaller body stores.

The Committee on Animal Nutrition of the National Research Council ('53) has established a maintenance requirement for adult dogs of 264 mg Ca/kg body weight/day, a figure approximately 20 times the recommended allowance for adult man. The same report notes that a diet which supplies about 132 mg/kg body weight/day appears to be adequate. This appears to be another reflection of "the more the better" attitude so common among nutritionists. In view of the greater stability of bone calcium resulting from adaptation to low calcium diets which appears to persist into old age (Henry and Kon, '53; Malm et al., '55) and the well known tendency for old men and animals to lose body calcium, it is possible to suggest that the latter tendency may be partly due to too liberal calcium intakes during early life.

Since we do not believe that the calcium requirement for balance is an indication of a real need, we cannot pretend to utilize the same technique to demonstrate a low-calcium requirement after adaptation to a low-calcium diet. However, when such data are combined with a complete absence of pathology indicative of a calcium deficiency, there is little foundation left for recommending intakes that are appreciably higher.

A retarded rate of bone development is common in children in at least some areas where calcium intakes are low (Trulson et al., '56; Walker, '54; Higginson, '54; Gillman and Gillman, '51; Jones and Dean, '56). There is as yet no evidence that such retardation is due to calcium inadequacy nor that calcium supplements would be useful. If there are benefits to be derived from the high-calcium intakes commonly recommended, they still require convincing demonstration.

SUMMARY

Three groups of two dogs each were reared and maintained on diets containing different levels of calcium. Balance studies showed that during the active growth period, those upon the

low-calcium diet (0.114%) retained calcium much more efficiently than those fed higher levels.

Repeated balance studies at different levels of calcium intake after the animals were 9 to 36 months of age demonstrated that the amount of calcium needed to maintain balance is largely a reflection of the previous calcium intake. The dogs "adapted" to a low-calcium diet had a calcium requirement for balance so low it could not be measured with accuracy and were not in consistent negative balance upon diets containing 0.034% of calcium.

Chemical and histological examination of the bones failed to reveal differences in composition or abnormalities attributable to calcium deficiency.

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CHOLESTEROL IN BLOOD AND TISSUES OF ADULT PANTOTHENIC ACID-DEFICIENT RATS [1]

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Pantothenic acid deficiency in weanling rats has been studied by many workers including Drell and Dunn ('51) who used an antagonist, ω-methyl pantothenic acid [2] to induce the deficiency syndrome. It is, however, more difficult to achieve this deficiency in the older rat (Novelli, '53). Bean and Hodges ('54) and Bean, Hodges and Daum ('55), using a synthetic diet lacking in pantothenic acid and containing this same antagonist, were able to produce symptoms of pantothenic acid deficiency in adult human males. Therefore it seemed of interest to apply this technique to the adult rat. In the present investigation adult males were used to study the effect of a pantothenic acid deficiency on cholesterol in both blood and tissues.

EXPERIMENTAL

Fifty-three male albino rats 3½ to 4 months of age weighing between 275 and 425 gm were used in three trials. Animals in trial 1 were of an inbred strain from the Department of Home Economics of the State University of Iowa. In trial 2

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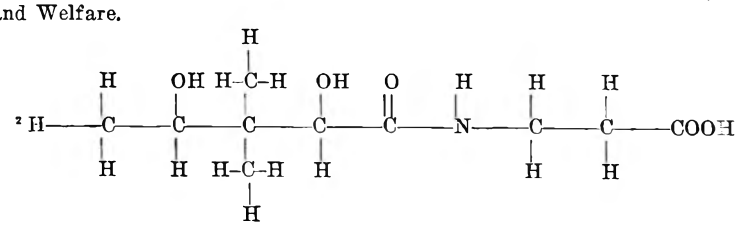

rats were of the Sprague-Dawley strain [3] and in trial 3 from Holtzman.[4] The rats were housed separately in metal, screen bottom cages. Food and water were supplied ad libitum in trial 1 but animals were pair fed in the usual manner in trials 2 and 3. Prior to the experimental periods all animals were maintained on a pellet chow.[5] The composition of the control diet in per cent was as follows: cornstarch 19, sucrose 39.8, vitamin free casein [6] 20, corn oil [7] 12, cod liver oil 3, mineral salts [8] 4, and vitamin mixture [9] 2.2. Pantothenic acid was omitted to form the pantothenic acid-deficient diet and the metabolic antagonist, ω-methyl pantothenic acid was combined with the ingredients, using 1 mg per 100 gm of food.

Duplicate determinations on whole blood were carried out weekly by drawing 0.1 ml samples from the tail vein. Cholesterol was extracted from the blood using an extracting fluid of equal amounts by volume of absolute alcohol, ether and acetone and determinations were made by the Leibermann-Burchard reaction using a modification of the Bloor technique (Sackett, '25).

At the end of 6 to 8 weeks, the experimental animals maintained on the pantothenic acid-deficient diet, with antagonist added, showed signs of deficiency.

Both the deficient rats and their control mates were then sacrificed by etherization.

Tissues were frozen until such time as analyses could be made. After thawing and weighing, tissues were homogenized

[3] Madison, Wisconsin.

[4] Madison, Wisconsin.

[5] Rockland Rat Diet, Arcady Farms Milling Company, Chicago, Illinois.

[6] Labco, The Borden Company.

[7] Mazola.

[8] Wesson modification of the Osborne and Mendel salt mixture, Nutritional Biochemicals Corporation.

[9] The vitamin mixture of pure vitamins triturated in glucose supplied the following amounts per 100 gm of diet for the control animals: α-tocopheral 11 mg, ascorbic acid 99 mg, inositol 11 mg, choline chloride 165 mg, menadione 4.9 mg, *P*-aminobenzoic acid 11 mg, niacin 9.9 mg, riboflavin 2.2 mg, pyridoxine 2.2 mg, thiamine hydrochloride 2.2 mg, calcium pantothenate 6.6 mg, biotin 44μg, folic acid 198μg, vitamin B_{12} 2.9μg.

in glass homogenizing tubes and cholesterol was extracted using the extracting fluid. Total cholesterol was determined on duplicate homogenates in the same manner as the determinations on blood.

RESULTS

With the use of a diet containing the metabolic antagonist, ω-methyl pantothenic acid, symptoms of pantothenic acid deficiency appeared in adult male rats after 6 to 8 weeks. In the deficiency state the animals exhibited loss of weight, bloody whiskers, soreness around the nose, respiratory infection and

TABLE 1

Average weights of rats on control and pantothenic acid-deficient diets

WEEKS ON DIET	TRIAL II [1]		TRIAL III [2]	
	Control	Deficient	Control	Deficient
	gm	*gm*	*gm*	*gm*
1	314	329	406	406
2	322	331	432	443
3	343	319	450	436
4	334	310	445	426
5	341	290	445	402
6	339	278	457	382
7	339	272	458	375
8	335	258	429	350

[1] Average of 10 control and 10 deficient animals.
[2] Average of 7 control and 7 deficient animals.

thinning and discoloration of the fur. Six of the animals died suddenly before they were sacrificed. Data for weights of animals for trials 2 and 3 are shown in table 1. Control animals gained the first two weeks and after that maintained their weight until the last week. Deficient animals after the first two weeks showed a drop in weight.

Data for total cholesterol in whole blood are shown in figure 1 for trials 2 and 3. Levels for trial 1 were essentially the same. There was a slight lowering during the experimental period for both control and deficient animals. This can probably be attributed to the fact that the fat used in the

diet was corn oil which has been found by Beveridge et al. ('56) to lead to a depression of plasma cholesterol levels. Corn oil contains sitosterols (Rathmann, '57), which have been reported by Best et al. ('54) as being only slightly absorbed, and therefore may interfere with absorption of cholesterol and thus cause significant reductions in serum cholesterol levels. Some variation was found in blood values from one week to the next and also variation between animals. However, when mean weekly values of deficient animals for each trial were compared with control animals no differences were found. Blood cholesterol values are slightly higher in this study than

Fig. 1 Blood cholesterol values of control and experimental rats fed a diet lacking in pantothenic acid with a metabolic antagonist ω-methyl pantothenic acid added.

have been reported by some workers. The extracting fluid of equal parts by volume of absolute alcohol, ether and acetone suggested by Lodigiani ('47) and Kier ('51) [10] was found to be superior to other solvents for cholesterol extraction.

Data for cholesterol in tissues are found in table 2. The largest amounts of cholesterol in both control and experimental tissues were found in the adrenal glands. Significantly smaller amounts were found in lung followed by spleen and liver. When cholesterol levels from experimental animals were compared with those in tissues from control animals differences were not apparent except for adrenal glands, in which the differences were found to be significant at the

[10] L. C. Kier, 1951, unpublished data.

TABLE 2

Total cholesterol content of tissues of control and pantothenic acid-deficient rats

	NO. OF ANIMALS	LIVER	SPLEEN	LUNG	ADRENAL		
		% Moist weight	% Moist weight	% Moist weight	Wt. of gland	% Moist weight	MG/Adrenal
					mg		
			Trial 1				
Food ad libitum							
Control	7	0.31 ± 0.05 [1]				5.37 ± 1.74	
Deficient	12	0.31 ± 0.04				3.10 ± 1.34	
			Trial 2				
Pair fed							
Control	10	0.31 ± 0.02	0.47 ± 0.09	0.55 ± 0.05	18 ± 3.7	6.80 ± 1.5	1.24 ± 0.12
Deficient	10	0.29 ± 0.04	0.46 ± 0.04	0.53 ± 0.05	18 ± 5.0	3.26 ± 0.8	0.53 ± 0.14
			Trial 3				
Pair fed							
Control	7	0.34 ± 0.05	0.42 ± 0.02	0.49 ± 0.02	25 ± 5.3	7.09 ± 0.83	1.76 ± 0.46
Deficient	7	0.30 ± 0.06	0.41 ± 0.02	0.50 ± 0.04	66 ± 31.5	1.13 ± 0.42	0.69 ± 0.32

[1] Mean and standard deviation.

1% level of confidence. The adrenal glands from all deficient animals at the time of autopsy appeared grossly hemorrhagic and in trial 3 there was also enlargement. Microscopic examination disclosed massive hemorrhagic necrosis of the adrenal cortex. Macrophages admixed with proliferating fibroblasts were noted in the capsular region, and in focal areas extending into periadrenal fat. Greater chemical and histological changes were evident in adrenal glands in trial 3 than in the other trials and it is assumed there was a greater degree of deficiency.

SUMMARY

Pantothenic acid deficiency was produced in albino rats 3½ to 4 months of age in 6 to 8 weeks using a pantothenic acid-deficient diet and a metabolic antagonist ω-methyl pantothenic acid.

There was a slight lowering of blood cholesterol for both control and deficient animals during the experimental period probably due to the use of corn oil in the diet. When mean weekly values for deficient animals were compared with those for control animals no differences were found.

Lower cholesterol levels were found in adrenal glands from deficient animals than in those from control animals but significant changes were not shown in lung, liver or spleen. Histological examination of adrenal glands from deficient animals showed massive hemorrhagic necrosis of the adrenal cortex.

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